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SPECIAL ANALYTICAL TECHNIQUES IN ENVIRONMENTAL MEDIA - THIN LAYER CHROMATOGRAPHY. TRAINING COURSE MANUAL

Environmental Control Administration
Cincinnati, Ohio

February 1970

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TRAINING COURSE MANUAL

SPECIAL ANALYTICAL TECHNIQUES IN ENVIRONMENTAL MEDIA - THIN LAYER CHROMATOGRAPHY

NATIONAL TECHNICAL
INFORMATION SERVICE

U.S. DEPARTMENT OF HEALTH, EDUCATION, & WELFARE
Public Health Service
Consumer Protection & Environmental Health Service

SPECIAL ANALYTICAL TECHNIQUES IN ENVIRONMENTAL MEDIA - THIN LAYER CHROMATOGRAPHY

This course is designed for technically trained personnel in control and regulatory laboratories concerned with problems associated with food protection, occupational health, and water hygiene.

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U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE
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Cincinnati, Ohio 45213

February 1970

SPECIAL ANALYTICAL TECHNIQUES IN ENVIRONMENTAL MEDIA -
THIN-LAYER CHROMATOGRAPHY
February 2 - 4, 1969
AGENDA

Moderator: J. D. Pfaff

DAY AND TIME	SUBJECT	SPEAKER
Monday, February 2		
9:00 - 9:15	Registration	
9:15 - 9:30	Welcome	J. D. Pfaff
9:30 - 9:45	Course Introduction	G. R. Shultz
9:45 - 10:00	Break	J. D. Pfaff
10:00 - 11:00	Fundamentals of T. L. C.	
11:00 - 12:00	Fundamentals of Paper and Column Chromatography	P. F. Hallback
12:00 - 1:00	Lunch	J. D. Pfaff
1:00 - 2:00	Choosing the Sorbent and Solvent	
2:00 - 3:30	Laboratory	T. W. Stanley
3:30 - 4:30	Chromatography of P. N. H. C.	Staff
		T. W. Stanley
Tuesday, February 3		
8:30 - 9:15	Comparison of Methods	
9:15 - 9:30	Break	J. D. Pfaff
9:30 - 10:30	Chromatography of Pesticides	
10:30 - 12:00	Laboratory (pesticides)	S. N. Dereniuk
12:00 - 1:00	Lunch	Staff
1:00 - 4:30	Laboratory (pesticides)	
		Staff
Wednesday, February 4		
8:30 - 9:15	Spot Detection and Plate Documentation	S. N. Dereniuk
9:15 - 9:30	Break	
9:30 - 10:30	Laboratory (two dimensional)	Staff
10:30 - 11:30	Special Tecniques	J. D. Pfaff
11:30 - 12:15	Qualitative and Quantitative	S. N. Dereniuk
12:15 - 1:30	Lunch	
1:30 - 2:30	Laboratory (two dimensional)	Staff
2:30 - 3:00	Discussion of Lab Results	Staff
3:00 - 4:30	Closing	J. D. Pfaff

SPEAKERS

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SECTION I

FUNDAMENTALS OF THIN-LAYER CHROMATOGRAPHY

FUNDAMENTALS OF THIN-LAYER CHROMATOGRAPHY

R. A. Simon*

I INTRODUCTION

TLC is a type of chromatography which is carried out on thin layers of adsorbent materials which have been spread on a suitable support. TLC supplements, and to some extent replaces column, paper and ion-exchange chromatography.

The standard procedure may be thought of as consisting of three steps: preparation of the plates, spotting and development of the sample and identification of the separated substances.

A slurry is prepared by mixing the powdered adsorbent with a suitable liquid; this mixiure is then spread in a thin layer on the plate using a special applicator. The plate is allowed to dry. Many of the adsorbents contain a binder to improve adhesion to the plate.

The sample is then spotted near the bottom of the plate, and the spotted plate is placed in a tank containing the chromatographic solvent. The sample spots are at the bottom of the tank, just above the solvent level. Development usually occurs by the ascending method.

Detection, if the spots are not already visible, and identification are accomplished by chemical reaction, by charring or by examination under ultra-violet light. Separated components may be removed from the chromatoplate for further examination.

II PREPARATION OF THE CHROMATOPLATES

A plate is prepared by spreading a thin layer of adsorbent material on it. The plate with the adsorbent layer is referred to as the chromatoplate.

A Adsorbent

The adsorbents used are generally those used in column chromatography. They are almost exclusively oxides, hydrated oxides or salts. Activity of these adsorbents varies widely, and a suitable adsorbent must be chosen for each desired separation.

Silica gel is the adsorbent most frequently used. It is probably the most versatile, and is readily obtainable.

Others which have been used are alumina, activated charcoal, cellulose, acetylated cellulose, kieselguhr and polyamide.

B Binder

Originally, no binder was used, but the resulting plates were fragile and flaked easily. More recently, plaster of Paris ($CaSO_4$) has been used to bind the adsorbent layer. Its inorganic properties allow treatment with even harsh reagents. Starch is also a good binder, but much less resistant to chemical attack.

Many of the commerical adsorbents already contain a binder, usually $CaSO_4$. These are always specified by the manufacturer. An example of this type is Silica Gel G; the "G" indicates "gypsum", one form of $CaSO_4$.

Some of the separations now being done employ no binder, e.g., the separation of the benzpyrene fraction on cellulose acetate. These layers are definitely fragile and require careful handling.

C Preparation

The adsorbent and the binder (if any) are mixed or shaken with a suitable liquid, and this slurry is applied to the plates.

In the standard method, glass plates 10 × 20 cm or 20 × 20 cm are used. These may be replaced by larger or smaller glass plates, microscope slides, metal plates or plastic sheets. Practically any support which will hold the layer and will not react with the solvent could conceivably be used.

The adsorbent slurry may be spread on the plates in any of a number of ways. The best method is to use one of the applicators specially made for this purpose. These differ in design from one manufacturer to another, but the principle remains the same. There is some arrangment for spreading

*Chemist

the adsorbent in a layer of regular, known thickness. These may provide layers of one fixed thickness (Desaga model 600) (see Figure 1) or the layer thickness may be adjustable (Desaga model 611) (see Figure 2). With both of the above types, the plates are placed on an aligning tray, and the spreader is drawn over the series of plates at a regular speed. (see Figure 3)

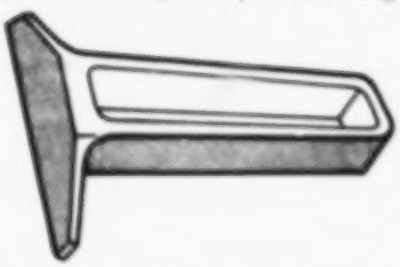

Figure 1 - Desaga Model 600 Spreader

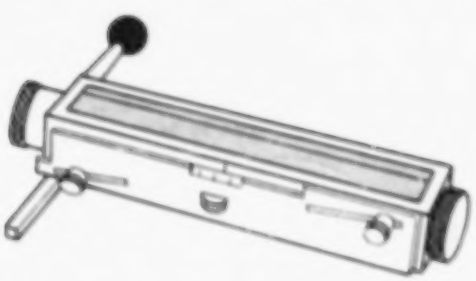

Figure 2 - Desaga Model 611 Spreader

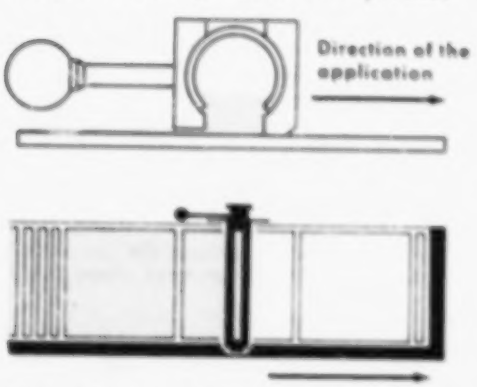

Figure 3 - Operation of the Applicator. Top: Functioning of the Applicator. Bottom: Template with Slides. Those to the Left of the Applicator Have Already Been Covered.

Other applicators vary according to the means of coating the plates. The Camag apparatus (see Figure 4) consists of an adjustable spreader mounted on a baseboard. The plates are pushed forward under the spreader by means of the succeeding plate.

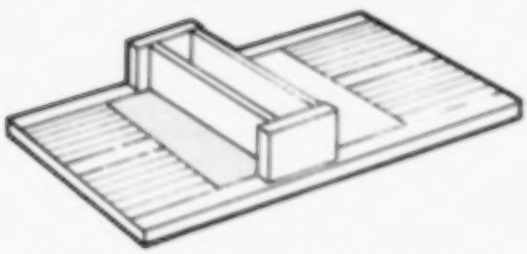

Figure 4 - Camag 3092-C10 Applicator for 200 and 100 mm Plates

The two types mentioned above are probably those most widely used. Other methods include use of a plastic coating rod (Serva-Entwicklungslabor Co.), an inflatable base to level the plates (Unoplan), a glass rod with thickened ends, or even a spatula. In general, the best results are obtained when spreaders specially designed for this purpose are used.

Irrespective of the method employed, a few basic principles must be observed. It is of utmost importance that the plates be clean and grease-free. They may be scrubbed with scouring powder, or soaked in chromic acid cleaning solution, detergents or concentrated sodium hydroxide. They are then rinsed thoroughly and dried.

The clean plates are then spread with the adsorbent as described above and allowed to dry. For lipo-soluble substances the plates must be activated by heating. After activation the plates should be stored in a desiccator until use.

III SPOTTING AND DEVELOPMENT

A Spotting the Sample

In TLC the sample is applied to the plate much as in paper chromatography. The simplest method is the use of a capillary tube. A small amount of the sample (dissolved in a suitable solvent) is allowed to rise in the capillary. The capillary is

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then touched lightly to the layer, about 1.5 - 2 cm from the bottom edge; the sample is thus transferred to the plate. Standards are usually applied in the same manner directly on the sample plate.

If a greater sample volume is needed, the process is repeated allowing the spot to dry completely between applications. Applying small volumes each time is essential for keeping the spot diameter small; large spots increase diffusion of the sample, giving a less well-defined separation.

The obvious problem with using capillaries for sample application is the lack of quantitation. The volume spotted is not accurately known; therefore, a quantitative analysis is impossible.

For quantitative results suitable micro pipets are used. These usually transfer a sample volume of a few μl. The sample is spotted in the same manner.

Micro syringes can also be used. Care must be taken to insure that the layer is not scratched. Positive volume control is possible with these syringes.

A wide-band pipet is available for use in preparative TLC; this technique discussed further in section IV E, below. The pipet transfers the sample as a rather wide band at right angles to the direction of development.

Again, a few basic principles apply to any of the spotting methods. The spot should be kept as small as possible. This is accomplished by applying the sample in repeated small volumes, allowing the spot to dry between applications.

The spots must be identified in some way before the separation begins. There is usually enough unused space for notes at the top of the plate. Complete identification should be recorded in the laboratory notebook.

Templates are available for use in spotting the plates, and for later calculating R_f values.

B Developing the Chromatogram

After the plate has been spotted it is placed in the chromatography tank. This tank may be of any of several designs. Basically, it must be large enough to hold the plates, with provision for closing the tank tightly.

It is often advisable to line the tank with filter paper which dips into the solvent. This assures a saturated atmosphere.

The choice of solvent will depend upon the separation desired. It is generally best to use solvents which are specified as "chromatographic quality," since purity is of utmost importance.

C Type of Development

Most TLC is done by ascending development. In this type the solvent is placed in the tank to a depth of perhaps 1 cm. The plate is then simply lowered into the tank, with the lower end immersed in the solvent, but not covering the spots. The solvent is allowed to rise by capillary action up through the plate, and the separation occurs along the line of development.

Two dimensional chromatography can also be done. Standards then are spotted in both directions.

Some separations are done by descending or horizontal development, but each of these requires some specialized equipment or adaptations.

IV ADVANCES AND MODIFICATIONS

A Prepared Plates

Commercially prepared plates are now available from several suppliers. Most of the commonly used adsorbents are stocked in the usual thicknesses. Virtually any other adsorbent can be specially ordered in a thickness to suit the need. (Analtech "Uniplate"; Brinkman Precoated TLC Plates).

These commercial plates are mechanically prepared so that the layer thickness is very consistent. They are especially useful when TLC work is done only occasionally, or in any situation in which preparation time is limited. The cost of prepared plates, although slightly higher than the cost of time and materials to prepare them in the laboratory, is not prohibitive.

They may also be obtained on special types of plates, e.g., ridged plates, or specially adapted, e.g., pre-scored layers.

B Sheets

In addition to prepared plates other newer types of TLC media are available. The layers may be fixed to a flexible plastic sheet from which zones may easily be cut for further manipulation (Eastman Chromagram).

Sheets of a paper-like TLC adsorbent can also be purchased. It consists of glass filaments combined with the adsorbents. This looks and acts very much like the filter paper in paper chromatography, although the mechanism of separation is different. It has the advantages of being easily cut to desired size and shape, and of being convenient to store (Gelman ITLC Applied Science Labs inc.)

C Simplified TLC Kits

Newer, less sophisticated setups for plate preparation are now in existence. One of these depends upon a standard-thickness tape to regulate the thickness of the layer. The tape is placed along two edges of the plate. (see Figure 5) The slurry is poured on, then spread by resting a glass stirring rod on the tape and drawing it down the plate. (see Figure 6)

Figure 5 - Glass Plate with Tape in Place (Chromakit)

Figure 6 - Spreading the Adsorbent with a glass stirring rod (Chromakit)

The initial cost of this kit is quite low, and contains everything necessary for layer preparation (Mallinkrodt Chroma-Kit).

D Preparative Chromatography

Although basically an analytical tool, TLC may be used as a preparative procedure. Generally, for preparative chromatography thicker layers (up to 2 mm) are desirable. The sample is applied as a streak rather than as discreet spots (see Figure 7). Special pipets are available for this purpose (Desaga Wide-Band Streaking Pipet) (see Figure 8), or mechanical devices may be used (Desaga Micro Spray Pistol or Desaga Electromechanical Sample Applicator).

Figure 7 - Sample Streak in Preparative TLC

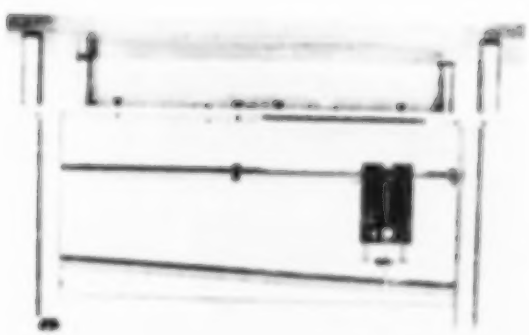

Figure 8 - Desaga Wide-Band Streaking Pipet

Because relatively large amounts of sample are involved, after separation the compounds of interest may be scraped off the chromatogram, and further manipulations may be performed.

V ADVANTAGES OF TLC

A Development is much more rapid than in paper or column chromatography. The average developing time with standard solvents at a thickness of 275 μ is from 20 - 40 minutes.

B The use of inorganic layers permits treatment with harsh reagents for detection of spots.

C Sharper separations are possible than those obtained by column or paper chromatography.

D Very small quantities of sample can be separated; usually a few thousandths of a milligram is sufficient. This is from 10 - 100 times less than is required for paper chromatography.

E Volume of sample per spot may vary from as little 0.5μg to as much as 500μg. This provides for a wide variety of applications.

F Results may be correlated to column separations because the same materials are generally used in both techniques.

G Equipment required is relatively inexpensive when compared with other separatory techniques.

COMPOUND	ADSORBENT	REFERENCE
Acids Carboxylic Acids	Silica Gel, $CaSO_4$ binder	Wollrab, et al, Collection Czech. Chem. Commun. 28, 1316 (1963)
Aliphatic and Aromatic Carboxylic Acids	Silica gel, $CaSO_4$ binder	Braun and Geenen, J. Chromatog. 7, 56 (1962)
n-Monocarboxylic Acids C_4 to C_{10}	Silica gel, $CaSO_4$ binder	Hromatka and Aue, Mh. Chem. 93, 503 (1962)
Isomeric Dicarboxylic Acids	Silica gel, $CaSO_4$ binder	Petrowitz and Pastuska, J. Chromatog. 7, 128 (1962)
Dicarboxylic Acids I	Kieselguhr, $CaSO_4$ binder, impregnated with polyethylene	Knappe and Peteri, Z. Anal. Chem. 188, 184 (1962)
Aromatic Acids	Gelatin: Silica gel, $CaSO_4$ binder; Polyamide (9, 5-7-4); Polyacrylonitrile: Polyamide (1:4)	Burkofer et al., Z. Naturforsch, 17b, 352 (1962)
ALCOHOLS, PHENOLS AND DERIVATIVES Alcohols	Silica gel	Bergelson et al., Doklady Akad. Nauk S.S.S.R. 141, 84 (1961); C.A. 56, 8005 (1962)
Aliphatic Alcohols, Glycols and Diketones	Aluminum Oxide	Cuevas J., Collection Czech. Chem. Commun. 28, 1341 (1963)
Long-chain Aliphatic Alcohols	Silica gel, $CaSO_4$ binder	Subbarao et al., J. Chromatog. 9, 295 (1962)
Acetylene Alcohols and Glycols	Al_2O_3	Akhrem et al., Invest. Akad. Nauk S.S.S.R. Otdel. Khim. Nauk 657 (1962)
Glycol, glycerols	Silica gel, $CaSO_4$ binder	Frey et al., Mikrochim. Acta, 499 (1962)
Phenols and Glycols, Nitro and Amino Phenols	Aluminum Oxide	Hermanek et al., Pharmazie 16, 566 (1961)

Some Applications of TLC to Analysis of Potential Atmospheric Pollutants continued

COMPOUND	ADSORBENT	REFERENCE
Phenols	Polyamide	Lin et al., J. Chinese Chem. Soc. (Taiwan) 9, 68 (1962)
		Wang, K. T., J. Chinese Chem. Soc. (Taiwan) 8, 241 (1961)
Phenol derivatives	Silica gel	Lymann et al., J. Org. Chem. 23, 756 (1958)
Phenols	Silica gel impreg. with oxalic acid	Furukawa, T., Nippon, Kagaku Zasshi 80, 387 (1959); C.A. 54, 13938 (1960)
	Silica gel, starch binder; Silica gel, starch binder: Kiesel guhr (1:1)	Wagner, G., Pharmazie 10, 302 (1955)
Homologous phenols	Silica gel, $CaSO_4$ binder; Polyamide	Halmekoski, J. and Hannikaninen, H., Suomen Kemistliehti B36, 24 (1963); C. A. 59, 8c (1963)
Tar constituents	Silica gel, $CaSO_4$ binder	Sensi et al., J. Chromatog. 5, 519 (1961)
		Stahl, E., Parfuemerie u. Kosmetik 39, 564 (1958)
Tar Oils	Silica Gel, $CaSO_4$ binder	Petrowitz, H.-J., Materialpruefung 2, 309 (1960)
Various phenols, Phenol Carboxylic Acids	Silica gel, $CaSO_4$ binder	Pastuska, G., Anal. Chem. 179, 355 (1961)
Phenolic Aldehydes	Silica gel, $CaSO_4$ binder	Sundt and Saccardi, Food Tehnol. 16, 80 (1962)
Phenols and Phenol Ethers	Silica gel, $CaSO_4$ binder	Klouwen and terHeide, Parfuemerie Kosmetik 43, 195 (1962)
ALDEHYDES, KETONES AND DERIVATIVES Unsaturated aliphatic aldehydes and acyloins	Silica gel, $CaSO_4$ binder	Gauglitz and Malins, J. Am. Oil Chemists' Soc. 37, 425 (1960)
Long-Chain aliphatic Ketones and aldehydes	Silica gel, $CaSO_4$ binder	Marcuse, R., J. Chromatog. 7, 407 (1962)
B-dicarbonyl compounds	Silica gel, starch binder	Brabier et al., Izvest. Akad. Nauk S. S. S. R. Otdel. Khim. Nauk 162, (1961); C. A. 55, 16077 (1961)

Some Applications of TLC to Analysis of Potential Atmospheric Pollutants continued

COMPOUND	ADSORBENT	REFERENCE
Acetals	Silica gel, $CaSO_4$ binder	Kore et al., Masloboino-Zhirovaya Prom. 28, no. 3. 32 (1962)
Aromatic aldehydes	Silica gel, $CaSO_4$ binder	Klouwen et al., Fette, Seifen, Anstrichmittel 65, 414 (1963)
Aliphatic and aromatic aldehydes and ketones	Aluminum oxide	Hermanek et al., Z. Pharmazie 16, 566 (1961)
Aromatic aldehydes and Ketones	Silica gel, $CaSO_4$ binder	Furukawa, T., Nippon Kagaku Zasshi 80, 387 (1959); C. A. 54, 13938 (1960)
AMINES AND INDOLES amines	Silica gel, $CaSO_4$ binder Cellulose, normal, Buffered silica gel, $CaSO_4$ binder	Teichert et al., Deut. Apotheker Ztg. 100, 283 (1960)
	Silica gel, $CaSO_4$ binder impreg. with boric acid	Pastuska and Trinks, Chem. Ztg. 86, 135 (1962)
Aromatic amines and nitro compounds	Silica gel, $CaSO_4$ binder	Furukawa, T., Mem. Fac. Educ., Hiroshima Univ. 5, 85 (1957); C. A. 52, 13364 (1958)
Aromatic amines	Silica gel, $CaSO_4$ binder	Gillio-Tos et al., J. Chromatog. 13, 572 (1964)
HYDROCARBONS Hydrocarbons and polycyclic aromatic hydrocarbons	Cellulose, acetylated	Badger et al., J. Chromatography 10, 397 (1963)
Aromatic hydrocarbons and heterocyclic compounds	Silica gel, $CaSO_4$ binder Aluminum oxide, $CaSO_4$ binder	Kucharczyk et al., J. Chromatog. 11, 55, (1963)
Polynuclear compounds	Silica gel	Haub and Kaemerrer, J. Chromatog. 11, 487 (1963)
Polynuclear aromatics	Cellulose, acetylated	Determann et al., Experientia 18, 430 (1962)
Anthracene derivatives	Silica gel, $CaSO_4$ binder	Teichert et al., Z. Anal Chem. 181, 325, (1961)
Polynuclear Aza Hetero-cyclic compounds	Aluminum Oxide, Silica gel, Carboxymethylcellulose Silica gel, $CaSO_4$ binder Adsorbil Cellulose, Acetylated	Sawicki et al., Anal Chem. Acta 31, 359 (1964)

Some Applications of TLC to Analysis of Potential Atmospheric Pollutants continued

COMPOUND	ADSORBENT	REFERENCE
Olefins	$AgNO_3$ - Silica gel	Gupta and Dev, J. Chromatog. 12, 189 (1963)
MISCELLANEOUS ORGANICS		
Peroxides	Silica gel, starch binder	Maruyama, K., J. Chem. Soc. Japan, Pure Chem. Sect. 77, 1496 (1956)
Some azo and nitro compounds	Cellulose, normal	Knabe and Kraeuter, Arch. Pharm. 296, (1963) 190-205
PROTEINS		
Proteins	Polyamide bound hydroxylapatite	Hofmann, A. F., Biochem. Biophys. Acta 60, 458 (1962)
Proteins	Sephadex	Morris, C. J., Biochem. J. 92, 6P (1964); C.A. 61, 15018
SYNTHETIC AND NATURAL DYES		
Dyes in gasoline	Silica gel, $CaSO_4$ binder	Haeusser, H., Arch. Kriminol. 125, 72 (1960)

SECTION II

FUNDAMENTALS OF PAPER CHROMATOGRAPHY

FUNDAMENTALS OF PAPER CHROMATOGRAPHY

R. A. Simon

I INTRODUCTION

Paper chromatography is one of the simplest of the chromatographic techniques. In this type of chromatography the sample solution is applied as a spot near one end of a sheet of filter paper. A suitable solvent (or mixture of solvents) is allowed the flow along the paper. As the solvent moves past the spot various components of the sample mixture will be carried along with it. The different rates at which the various substituents are carried provide the basis for a separatory technique.

A The Paper

The filter paper in this type of chromatography usually acts only as a support for the liquid stationary solvent. This filter paper can be of any of the commercial types specified for chromatographic use.

B The Solvents

There are generally two solvents (or mixtures of solvents) involved in paper chromatography: the stationary solvent which is held on the filter paper support, and the mobile solvent which flows past the stationary phase.

This is basically a liquid-liquid extraction procedure. The extent to which a given solute travels is dependent upon its partition coefficient.

PARTITION COEFFICIENT = $a = C_1/C_2$

where C_1 is the concentration of solute in one phase, and C_2 is the concentration of solute in the second. This partition coefficient is constant for a given solvent system.

One general rule is usually followed: the two solvents should be "opposite" in polarity to one another. If the stationary solvent is polar, the mobile phase will be relatively non-polar; this is the usual situation. If a non-polar stationary phase is used, the mobile phase should be polar; this is referred to as "reverse-phase" chromatography.

C Sample Application

The sample is applied as in thin-layer chromatography. It is spotted on an origin line near one end of the paper by means of a capillary, a micropipet or a syringe. The spot should be as small as possible. If larger amounts of sample are to be applied, this should be done by drying the spot, then applying more sample solution at the same spot.

For preparative chromatography the sample is applied as a wide band, using a special streaking pipet.

II TECHNIQUES OF PAPER CHROMATOGRAPHY

The various methods of paper chromatography differentiated by the direction in which the solvent flows. A few of the more commonly used techniques will be considered briefly.

A Descending Chromatography

Descending paper chromatography is the type most often used. The stationary solvent is usually held in the bottom of the chromatography tank; when the tank is tightly sealed, the atmosphere within is saturated with this solvent. The mobile solvent is held in a trough near the top of the chamber. The end of the paper nearest the spots is dipped into this trough. The mobile solvent is allowed to flow downward through the paper. (see Figure 1)

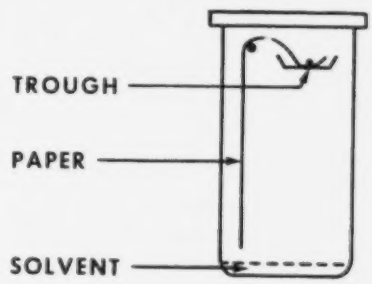

Figure 1 - Descending Chromatography

B Ascending Development

A technique which requires even less equipment is ascending development. A mixture of the two solvents is contained in the bottom of the chamber. The end of the paper near the spots is dipped into this solvent mixture, and the mobile solvent travels upward through the paper. (see Figure 2)

The paper may be suspended from the lid of the tank, or may simply be rolled into a cylinder and allowed to stand in the solvent.

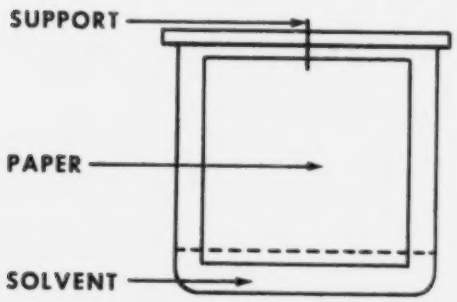

Figure 2 - Ascending Chromatography

C Radial Development

In radial development, the sample is spotted in the center of a circular piece of filter paper. The paper is held between two Petri dishes, the bottom dish contains the solvent. (see Figure 3)

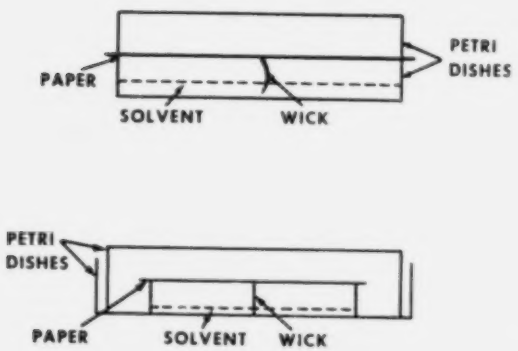

Figure 3 - Radial Development, Showing Petri Dish Arrangements

The solvent is fed to the paper through a wick and spreads radially through the paper and concentric annular rings are formed. (see Figure 4)

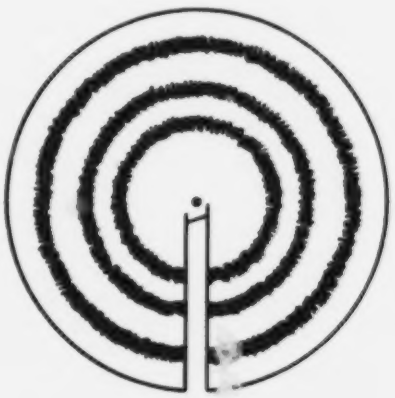

Figure 4 - Radial Chromatogram Showing Concentric Annular Rings

D Multiple Chromatography

To improve resolutions, multiple development techniques may be used, to perform two or more subsequent developments. The multiple technique most often used is two-dimensional chromatography. The sample is spotted near one corner of the paper, and is developed with the first solvent. Then the paper is rotated 90^{0} and is developed in the second solvent. (see Figure 5) Complex mixtures can often be resolved in this way.

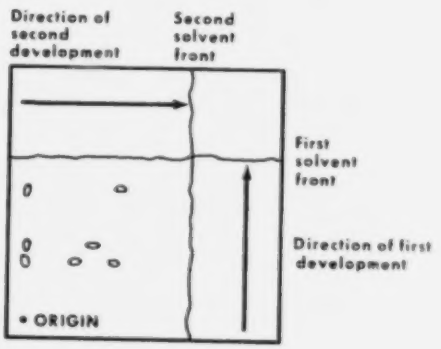

Figure 5 - Two Dimensional Chromatogram

E Other Techniques

Various other chromatographic techniques have been employed involving horizontal development, use of specially cut papers or modified papers, and more complicated equipment. These adaptations may be necessary for specialized applications, but those mentioned above should be adequate for routine separations.

III DETECTION

After development the spots may be detected by physical, chemical, biological or enzymatic means.

A Physical means include absorption of visible light (colored spots), ultra-violet light or infra-red light, or fluorescence of the spots.

B Chemical methods depend upon the formation of black, colored or fluorescent spots, by chemical reaction. The reagent is best applied by spraying.

C Spots may be detected by inhibition or acceleration of growth of suitable biological organisms. Enzymatic methods are used to detect enzymes and substrates.

IV QUALITATIVE AND QUANTITATIVE IDENTIFICATION

Although primarily a separation technique, paper chromatography may be used as an analytical tool. This can be accomplished in several ways.

A Chemical or Biological Reaction

When colored spots are formed, the color can indicate "what" is present, the size and intensity indicate "how much." The type and degree of biological activity also are qualitative and quantitative measures.

B. R_f

A good qualitative measurement is the R_f value. This is the ratio of the distance traveled by the spot to the distance traveled by the solvent front.

$$R_f = \frac{\text{distance traveled by center of spot}}{\text{distance traveled by solvent front}}$$

If all conditions are rigidly maintained, the R_f is constant for a given solvent system.

C Chromatographic Spectrum

A substance may be chromatographed in a series of solvents. A plot of the R_f's for the various solvents will be characteristic of the solute material, and is an almost certain means of identification. (see Figure 6)

Various other methods are used for identification. These may be performed while the spot is on the paper, or the spot may be removed from the paper, extracted and further analyzed.

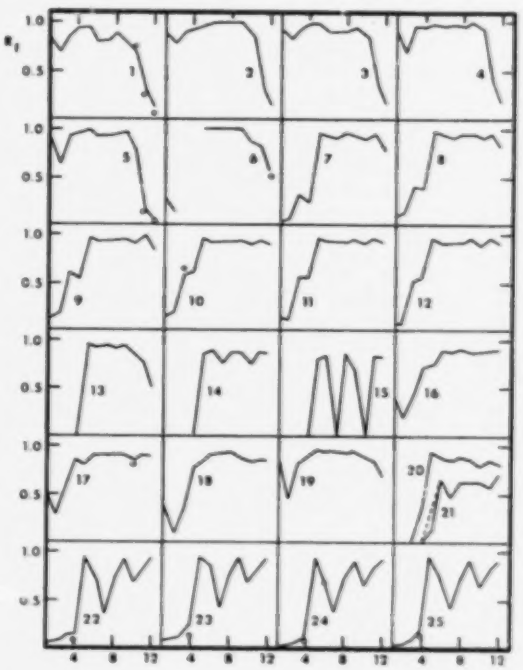

Figure 6 - Chromatographic Spectra (in 12 Solvent Systems)

1–Anthracene, 2–phenanthrene, 3–fluoranthrene 4–pyrene, 5–benzpyrene, 6–chrysene, 7–phenol, 8–p-cresol, 9–p-ethylphenol, 10–2, 3-dimethylphenol, 11-3, 5-dimethylphenol, 12–1-naphthol, 13–2-hydroxyanthraquinone, 14–resorcinol, 15-pyrogallol, 16–p-toluidine, 17–p-xylidine, 18–2-naphthylamine, 19–diphenylamine, 20–2-aminoanthraquinone, 21–p-phenylenediamine, 22–benzoic acid, 23–p-toluic acid, 24–phenylacetic acid, 25–cinnamic acid. (Reproduced from Franc, J., and Stránsky, Z., Coll. Czech. Chem. Communs. 24, 3611 (1959) by permission of the authors and of the Czech Acad. Science Publ. Co.)

V Advantages of Paper Chromatography

A Very little special equipment is required. Almost any container which can be tightly closed is a potential chromatography tank.

B The technique is relatively fast. The speed varies with the paper and solvent system, but 2-3 cm / hr might be a reasonable developing rate for ascending development. Although much slower than Thin-Layer Chromatography, this is much more rapid than other lengthy separation procedures.

C Good visual records are usually obtained. The finished chromatogram may easily be preserved and stored in a notebook or file.

D Paper chromatography has a wide variety of applications and can be used to separate both polar and non-polar materials.

VI SUMMARY

Paper chromatography is a separation technique which depends upon partitioning of the sample between two phases. The paper acts as a support. The sample is spotted on the paper and the solvent passes over it. After the spots have separated, various methods for detection and identification can be employed.

Many of the techniques used in paper chromatography are those used in Thin-Layer Chromatography. Reference may be made to the previous outline for further details on these.

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Pyrrole Acids	Chierici and Artusi, Ric. Sci., Rend., Sez. A 6, 170 (1964)
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SECTION III

FUNDAMENTALS OF COLUMN CHROMATOGRAPHY

FUNDAMENTALS AND APPLICATION OF COLUMN CHROMATOGRAPHY

John D. Pfaff[1]

I INTRODUCTION

Chromatography had its beginning in 1903, when Tsaett discovered that plant pigments could be separated by passing them through a column filled with calcium carbonate. Constant efforts to lower detectable limits and to generally improve the method led to the development of the other types of chromatography. Thus it can be said that column chromatography was the forerunner of all types of Chromatography.

Column chromatography is a simple and inexpensive method for the separation of mixtures. It usually consists of a cylindrical tube, the column, which is packed with an adsorbent, chosen to achieve the best possible separation of the mixture.

Larger amounts of sample, usually a half gram or more, must be used for column chromatography. This feature sometimes restricts the use of column chromatography for identification purposes. However, advantage has been taken of this fact to separate, prior to identification, large amounts of material. This is the basic idea of preparative chromatography.

The various methods of operating a column, ie. Frontal, Displacement and Elution, are covered in the outline on the "Theory of Chromatography." However, a brief definition of each is included here.

Column chromatograms may be developed by these methods:

A Frontal analysis, in which the original mixture of solvent and sample to be separated are continuously passed down the column.

B Elution development, in which only a small volume of the original mixture is at first placed on the column, by a pure solvent.

C Displacement development, in which a solution having more affinity for the columns packing is used to push or displace the mixture down the column.

II THE COLUMN

The most common type of columns are made of glass and are drawn to a narrow tip. In this narrow tip several small pieces of glass wool are placed. This glass wool serves as support for the adsorbent and also prevents the adsorbent from being washed out, while still allowing free passage of the solvent. It is desirable to use several small pieces of glass wool in place of a single piece. This is done in order to prevent channeling in the glass wool and subsequent loss of adsorbent.

Length and width of the column will effect the distance between separated materials. The separation obtained from a long narrow column will tend to leave more distance between bands, however, the material itself will be spread over a much larger area. In cases where separations are comparatively easy, it is however, possible to use shorter and wider columns which are often easier to pack and use.

The column may have many physical designs as shown in Figure 1. One of the more useful pieces of equipment would be a stop cock for adjusting and cutting off the flow of liquid (Figure 1c and Figure 1e). Many columns in place of the glass wool use a porous of perforated material as a support (Figure 1d). A container, attached to the upper end of the column (Figures 1b, 1c) is another labor saving device.

Figure 1

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Preparative chromatography has used columns which are split lengthwise. This permits the colum to be taken apart after it has been developed. The bands of material are then simply cut out and eluted. The two halves of the tube can be held together either with metal bands, tightened with screws, or some other manner.

If the flow rate desired is faster than the flow rate dictated by the length and degree of packing of the column, then the simple arrangement shown in Figure 1f is often quite satisfactory. By applying the solvent to the column under pressure, or at a given rate by pump the desired flow can also be obtained.

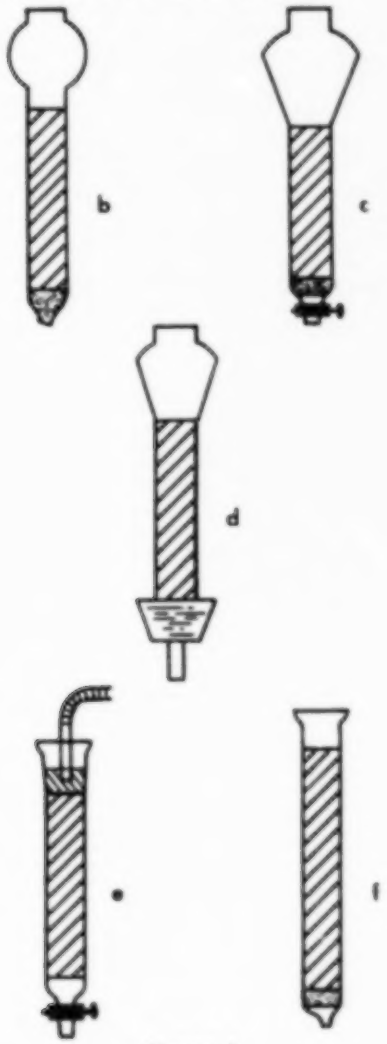

Figure 1

III FLOW RATE

The flow rate of the solvent through the column is critical in achieving maximum separation of the mixture. The usual rule followed is that the lower the flow rate the easier the separation of the mixture involved. This can be due in some instances to a sharpening of the leading edge of the separation. Flow rate and gram size of the adsorbent are dependent on each other. The larger the grain of the adsorbent the slower the flow must be in order to obtain good separation.

It may be said, when preparing a column that the best separation results will be obtained with a long and narrow column, filled with a fine-grained adsorbent and employing a low flow rate.

IV ADSORBENTS AND COLUMN PACKING

In principle all the adsorbents used for column chromatography can also be used for thin-layer chromatography. But since a very small average particle size is desirable in the latter case, the column materials must usually be ground finer.

The average particle size in thin-layer chromatography is 5 to 25μ, while the particle size used in a number of column chromatographic methods is approximately 75 to 125μ.

The most commonly used material in column chromatography is aluminum oxide, though other inorganic materials, eg. silica or charcoal, are frequently employed. Alumina is easily obtained and is capable of adsorbing an extremely wide range of types of solute, it is also colorless and organic materials are separated to a great extent by it.

Packing a chromatographic column can be done either wet or dry. When packing dry the adsorbent is poured into the tube in portions and tamped until it is well packed. After packing dry the adsorbent is poured into the tube in portions and tamped until it is well packed. After packing, the column must be washed with the solvent to be used, until all the trapped air is removed from the adsorbent.

In order to pack a column using the wet technique the adsorbent is slurried in the solvent and poured into the column. While the adsorbent settles to the bottom of the column, the solvent is allowed to flow out gradually. Fresh additions of the slurry are made to keep up with the outflow until all the material has been added.

This column must also be washed but with considerably less solvent. Wet packing is usually more popular than dry packing, because it is more convenient and gives more homogeneous columns.

V SAMPLE ADDITION

Care must be taken to insure an even addition of sample to a column. Especially since large amounts of sample are used in this type of chromatography. Uneven development will occur if the material to be separated is not put on the column correctly.

To achieve correct addition the sample is dissolved completely in a low boiling solvent which will not affect the sample. Then enough extra column adsorbent is added to the dissolved sample and stirred while the solvent is removed by evaporation. The resulting adsorbed sample is placed atop the column and development begun.

VI LOCATION OF BANDS

The location of the separated materials in column chromatography presents the user with a few more problems than with thin-layer or paper chromatography. The main difficulty is of course the glass column itself. If the material separated is colored or fluorescent, there is no problem. However, if the material is neither of these, another way would be to collect known amounts of the effluent and use some analytical tool to detect what materials are found in these various fractions.

If the column packing, with the separated mixture on it, can be removed from the column without breaking it up, it can be sprayed with a color forming reagent in order to visually locate the separated materials.

VII FRACTIONATION

Since the primary use of column chromatography has been a clean up or preparatory type of separation some method is needed to collect the effluent of the column. This need is satisfied by a fractionator Figure 2. The fractionator is an automatic device, capable of dividing the eluate into a number of fractions for further analysis or, simply for keeping separate the individual substances that have been separated.

Figure 2

Most fractionators are composed of a revolving wheel which holds many test tubes. The desired fraction from the column is collected in the tubes and removed for analysis.

Generally, it is desirable to obtain fractions of equal volume throughout the whole development of the column. This can be accomplished by the fractionator in several ways. The first way uses a present time interval for collection in each tube and a constant flow rate. However, since the flow resistance of a column often changes during operation a constant flow can be difficult to maintain, therefore, this method may not be practical.

Secondly, a siphon which automatically delivers a constant volume from the column to a test tube can be used. This is shown in Figure 3. The major drawback here is the volume cannot be changed easily except by changing the siphon itself.

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Figure 3

Using a phototube and constant drop size the number of drops falling into the collecting device can be determined. However, when a drop passes through the light of the photoelectric cell some of the mixture may be destroyed by photochemical reaction.

Other methods of collection may utilize weight of collected volume and timed flow collector. These methods necessitate a constant flow and collection devices which weigh the collected volume. These are expensive and the constant flow hard to obtain.

The fractionator using the phototube can be used except for materials which undergo photochemical changes. In this case a timed flow collector should be used.

VIII SUMMARY

Column chromatography has found its greatest application in Air Pollution in its ability to separate larger amounts of material into smaller fractions. This clean up or preparative use of column chromatography is a great advantage when identification of these materials is to be done.

Using this technique, materials present in trace amounts can be separated, concentrated and then identified. Identification is often accomplished by combining column chromatography with some analytical instrument such as a spectrophotometer or even combining it with a gas chromatograph.

The variables which can effect the separatory powers of a column are the length of a column, the degree and size of packing, the adsorbent and the solvents flow rate. These variables should be considered before the column is prepared and run.

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SECTION IV

CHOOSING THE SORBENT AND SOLVENT

SELECTION OF THE ADSORBENT AND SOLVENT

I INTRODUCTION

In chromatography, we are concerned with a system composed of at least three components, the adsorbent, solvent, and the compound being chromatographed. The behavior of the compound is dependent upon both the adsorbent and the solvent. The catalogue for one of the leading suppliers of adsorbents lists fourteen different types of adsorbents. With this many types of adsorbents available it is necessary to have some idea on the selection, for the students purpose, of the adsorbent.

While the choice of adsorbent is some what limited, the range of solvents and their mixtures can have a large variety. The student should be able to select the various right combination of adsorbent and solvent best suited to his needs.

II CHOICE OF STATIONARY PHASE

A Adsorbents for T. L. C.

The adsorbents used in thin-layer chromatography are those which can also be employed in column chromatography. These adsorbents are almost exclusively oxides, hydrated oxides or salts. When choosing adsorbents one can obtain some idea of which to choose by the characteristics of the materials to be separated. Consideration must be given as to solubility, acid or basic properties and if it will react with the adsorbent or solvent.

When the adsorbed materials are polar or polarizable compounds, they are bound to the surface of the adsorbent by the same forces which hold the crystallen lattice together. These surface forces induce dipole moments in nonpolar compounds and increase the existing dipole moments of polar compounds.

Therefore, adsorbents with a high adsorption activity should be used to separate unpolar compounds (hydrocarbons). Polar compounds (acids) should be separated on adsorbents with low adsorption activity. The polarity of the compound can be judged by the type of functional groups present. Table I lists the influence of functional groups on the polarity of a compound.

Table I

Influence of Some Functional Groups on the Polarity of a Compound

Functional group -	
- CH_3	
- o-Alkyl	
- c = o	
- NH_2	increasing polarity
- oH.	
- CooH.	

Acidic compounds (phenols, acids) can be separated only on an acidic or a neutral adsorbent and basic materials can be separated only on basic or neutral layers. The acidity of some of the adsorbents can be found in Table II.

Table II shows the adsorption activity for several of the more common adsorbents. However, by suitable pretreatment it is possible to obtain various degrees of activity. For example, silica gel shows the highest adsorption activity. If a less active material is required, one can decrease the adsorption activity either by exposing the layer to atmospheric humidity, by steaming the layer or by mixing silica gel with a less active adsorbent such as kieselguhr.

Any of the various reference works on chromatography will contain tables showing the activity of the various adsorbents. They will vary slightly usually by addition or deletion of some adsorbents.

Two of the major adsorbents (alumina and silica gel) must be activated Activation is carried out to drive off water which has filled the "active sites" of the adsorbent. These active sites are the areas which carry out the separation. In the process of activation an air dried plate is heated at a specified temperature for a set time. Once a procedure involving separation by thin-layer is developed all variables, such as activation must be carried out as reproducibly as possible. Thus, in the case of activation, the content of water adsorbed on the adsorbent can be controlled, and consequently the degree of activity of the thin-layer procedure, can be varied.

After a plate is activated, it should be allowed to cool in a desiccator and use as quickly as possible. If allowed to stand in the room air, the adsorption of water begins again. Therefore, activation can be an obstacle in the path of reproducibility in thin-layer procedures.

When a high degree of adsorption of water is reached it is often difficult to recognize whether a separation of compounds is due to adsorption or partition. It is possible in many cases that these effects will overlap.

Table II

Adsorbents

Adsorbent	Acidity
Acitvated Charcoal	
Silica Gel	Acidic
Aluminum Oxide	Basic
Magnesium Silicate	
Magnesium Oxide	
Calcium Phosphate	Weakly Basic
Calcium Carbonate	
Cellulose Powder	Neutral
Polyamides	Neutral
Kieselguhr	Neutral

Increasing Adsorption Activity

B Paper for Paper Chromatography

Although in most cases paper chromatography achieves separation by partition there are some instances where separation is achieved by adsorption. Adsorption in paper chromatography exists mostly when the paper has been impregnated with an adsorbent similar to those mentioned under thin-layer chromatography. In this case, the activation of the impregnated paper will correspond to Table II.

When partition chromatography is employed the paper acts as a support only. Partition chromatography occurs when distribution is achieved between a mobile liquid phase and a stationary liquid phase. Therefore, the choice of the paper is not as important as the choice of the two solvents.

II SELECTION OF THE SOLVENT SYSTEM

When choosing an adsorbent, one should differentiate between adsorption and partition chromatography. Adsorption is the simpler of the two because the solvents are completely miscible and their ability to move a sample is somewhat predictable. Partition chromatography, however, involves a selection of a two phase system which will have a coefficient necessary for separation.

A For Adsorption

Since the rate of migration of a compound on a given adsorbent depends on the solvent used a choice can often be made by consulting the eluotropic series. This series lists various solvents in order of elutive power (Table III). The elution power of solvents in adsorption chromatography has been explained by reference to a number of factors, e.g. polarity, dielectric constant, formation of hydrogen bonds. It should be pointed out that the order of elution power of a given set of solvents is not identical for all adsorbents. Therefore, the elutropic series should be used as a good guide for choosing a solvent for adsorption chromatography.

Table III

Eluotropic Series of Solvents According to Stahl

Solvent	Dielectric Constant (20°)	
n-Hexane	1.890	
Heptane	1.924	
Cyclohexane	2.023	
Carbon Tetrachloride	2.238	
Benzene	2.284	
Chloroform	4.806	
Diethylether	4.34	
Pyridine	12.3*	
Acetone	20.7*	
Ethanol	24.3*	
Methanol	33.62	
Water	80.37	

Increasing elutive power ↓

* @ 25°C.

Mixtures of two or three solvents of different polarity often gives better separation than a single solvent. Randerath shows an elutropic series of two component solvents. However, most analysts will spend more time looking in the literature for solvent systems than he will in determining these experimentally.

A quick method of determining the elutive power of a solvent for a particular set of compounds may be used. Several spots of a solution of the compounds to be separated are placed on the chosen adsorbent. When dry, a small drop of eluent is placed at the center of the spot. The degree of separation of the spot will indicate if it is a suitable solvent.

B For Partition

Methods based on partition chromatography are found in thin-layer chromatography. However, the best known form of partition chromatography is paper chromatography.

Chromatography on thin layers of cellulose are completely analogous to paper chromatography.

Many solvent systems can be found in the literature to deal with the majority of commonly occurring compounds. However, if the materials are not covered the solvent system must be chosen by experimentation. The solubility in the mobile and stationary phases will give a good indication of the separation. A low solubility of the substance in the mobile phase is indicated when the substance remains close to the origin. In such a case a solvent lower in Table III should be tried as the mobile phase. If on the other hand, the substance migrates with the solvent front, it is not sufficiently soluble in the stationary phase. Therefore, a solvent higher in Table III should be used as the mobile phase. Because of the complexity of the solvent system, it is likely that the literature will provide an easier source of information than random experimentation.

SECTION V

COMPARISON OF METHODS

COMPARISON OF THE VARIOUS TYPES OF CHROMATOGRAPHY

I INTRODUCTION

Much of the material found in this outline can be found in the previously covered material. The purpose of this outline, then is to act as a review of the preceding outlines as well as making a comparison between the various types of chromatography.

Although most types of chromatography have their basic concepts in common, each has its own individual characteristics. These characteristics are what make up the strong or weak points of the method. It is these characteristics which we will discuss in this outline.

II ADSORBENTS

The first consideration should be the adsorbent or stationary phase of the various types of chromatography. We have seen that thin-layer and column chromatography have a much larger choice of materials than paper chromatography. Thin-layer chromatography has at its disposal about fifteen different absorbents. Each of the fifteen have many variations which further increases the choice.

Thin-layer chromatography and column chromatography have a greater affinity for separation than paper chromatography due to this wider choice of adsorbents.

Paper chromatography has three different materials used in the production of paper i.e., cellulose, glass and viscose rayon. Cellulose is used in most cases. Cellulose has many variations including cellulose impregnated with some type of solid absorbent. This type of paper, however, lies on the border line as it is not known how the separation is effected by the addition of the solid adsorbent.

III SEPARATION

A Amounts Capable of Being Separated:

The amount of material necessary for separation will vary with the method of detection. However, we can still say that very small amounts of the material to be separated are needed when using thin-layer and paper chromatography. When comparing these two methods thin-layer has the higher sensitivity.

Thin-layer chromatography has been used to test the separatory power of a column method. It has been found that one can often scale up directly from the micro-method (T. L. C.) to the macro-method (column chromatography). Amounts as low as several microliters of substance can be separated with thin-layer where column chromatography uses amounts of approximately one gram.

When it is desirable to separate large amounts of material column chromatography is the desirable method. There is the possibility of using preparative T. L. C. for large amounts. Some difficulty is encountered in keeping the thickened layers from splitting. Although a preparative separation of several hundred milligrams of mixture can be undertaken by preparative T. L. C. on a series of plates, column chromatography is the method of choice for separation on a gram scale.

B Speed:

One of the greatest differences between the methods of thin-layer, column and paper chromatography is the time necessary to develop the chromatogram. Understanding that the time of development is dependent upon many variables it can still be shown that in most cases thin-layer chromatography is the faster method. Methods of separation having development times of about 30 minutes are common for thin-layer chromatography.

One variation of paper chromatography, i.e. circular paper chromatography is the closest in development time to thin-layer chromatography. This is run horizontally and capable of running only a small number of spots.

Column chromatography is hard to compare on this point because its use is primarily separation using larger amounts of material. There are also several more variables in column work which will not hold for paper or thin-layer chromatography.

C Sharpness of Spot:

Thin-layer chromatography has the ability to give, in most instances, a greater sharpness of separation. This ability is usually attributed to a lesser degree of spreading of the spots. As a spot migrates up a plate

or paper it is also spreading in a direction perpendicular to the direction of the solvent flow. This degree of spreading therefore causes the sharpness and size of the spot to vary. From this variance we see several of the advantages of the thin-layer system over paper system. It can be seen that if spreading of the spot is at a minimum then the concentration per unit area is at its maximum. Then regardless of the method of detection the detection threshold of chromatographed substances are lowered. This then is the idea behind the fact that thin-layer chromatography is usually more sensitive than paper chromatography.

This spreading also helps to explain the fact that, in general, separation is possible in T. L. C. over a shorter length of time, thereby giving a substantially reduced time of development.

D Reproducibility of Separation:

All types of chromatography, under the same set of conditions will give the same general separation. That is, the order of separation of a given set of spots will not change. However, when the distance traveled by the spot is compared to the distance from the origin the solvent has traveled, i.e., the R_f value, the reproducibility varies considerably.

Thin-layer chromatography has more factors which will effect the reproducibility of the R_f values. These factors are the:

1 Quality of the Layer Material

2 Degree of Activities of the Layer

3 Layer Thickness

4 Technique in Preparation

There are several more but are not mentioned here because they will effect paper-chromatography to the same extent.

If all factors effecting the reproducibility are taken into consideration, the R_f values for T. L. C. and P. C. will correspond approximately. From this it can be seen that T. L. C. usually will have a greater degree of latitude in duplicating R_f values than will P. C.

IV SPOT DETECTION

Paper and thin-layer chromatography are more advantageous when the detection of the separated material is considered Column chromatography because of being enclosed in the column has considerably fewer methods of detection available. If the separated material is to be eluted or collected, the analyst must remove the adsorbent from the column in order to achieve the desired result. This can lead to considerable losses unless precise techniques are employed.

When inorganic adsorbents are used, i.e., in thin-layer chromatography, much more aggressive reagents can be employed for the detection of the material. Certain detector methods can not be employed with paper-chromatography because they may destroy or charr the support material. Considering the total number of detection methods the analyst still has a large number available when using paper chromatography.

V SOLVENTS

When considering the type of solvents available, it is sufficient to say that the only type of chromatography with any restrictions would be paper chromatography. This restriction would be the same as that considered when choosing a method for detecting the separated materials. That is, no solvent system can be used which would cause deterioration in the paper itself.

VI SUMMARY

It is important to understand the various types of chromatography. The analyst cannot choose which type will best fulfill his needs unless complete understanding is obtained. Since each type has its own individual characteristics and since these characteristics are what will decide the analyst for or against using a particular type then these characteristics should be known and evaluated.

A chemist could choose thin-layer chromatography for its speed, paper chromatography for its better reproducibility, or possibly column chromatography for its ability to separate larger amounts of material. In general, however, it must be pointed out that by nature chromatography is separatory in nature and only secondarily quantitative.

SECTION VI

SPOT DETECTION AND PLATE DOCUMENTATION

SPOT DETECTION AND PLATE DOCUMENTATION

J. D. Pfaff*

I INTRODUCTION

After separation of a mixture has been accomplished by a chromatographic method some manner of detection of the separated materials must be found. Each type of chromatography presents a special set of problems for detection. It is, for this reason, that gas chromatography is more easily standardized and instrumentized than solution chromatography or electrochromatography.

Paper chromatography can be more easily documented than thin-layer chromatograms. This is partially due to the thin-layer of adsorbent being more sensitive to mechanical damage than the paper used in paper chromatography. Another reason favoring the documentation of a paper chromatogram is that generally the color of the spots fade more rapidly in T. L. C. than those in P. C.

The R_f values obtained in paper chromatography are usually more reproducible than those obtained in thin-layer. As a result, recording these values is not enough to completely identify a chromatogram.

No matter how good the separation of the material is, nothing is accomplished without some means of detection and documentation. Therefore, the various methods of detection and documentation, of the separated materials, for paper, thin-layer, and column chromatography will be covered in this outline.

II SPOT DETECTION

A distinction should be made here that detection of a spot must be accomplished in some manner before documentation can be carried out. Under the present heading a discussion of only the methods used to detect the spots or bands will be given. Documentation will be covered in the next section.

A Use of Physical Properties:

The simplest method for detecting a spot is if the spot has some physical property by which direct measurement can be made. One such method utilizes the color of the materials being separated. Visual detection is the easiest method available.

Another natural phenomina used is the separation of radioactive material. The sample may be material which has been labeled or radioactive isotopes. Detection can be accomplished by passing the plate in front of a Geiger counter equipped with a narrow slit to admit the radioaction. Thereby locating the spots as they pass.

B Use of Fluorescence:

Special methods must be devised for the detection of the spots which are not found by their natural color or other physical property.

Many compounds become visible by adsorption of fluorescence when the chromatogram is viewed under a short-wave or a long-wave ultraviolet lamp. One example of this is the detection of hydrocarbons after separation.

Several devices are available which can be used for the viewing of the chromatogram by ultraviolet light. The simplest device is shown in Figure 1. This lamp has only the long or short wave lengths so two lamps must be used in order to cover the complete range.

Figure 1

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Another device which is of much more use is a box which can keep out room light and comes equipped with both long, short and visible lamps. Other devices are available which use transmitted UV light and can be used with a cabinet. This set up can detect material which has only weak fluorescence and which would probably be missed when viewed in room light by a lamp such as shown in Figure 1.

Another method in use utilizes the lack of fluorescence. Organic compounds which absorb light in the UV range can be visualized by spraying the chromatogram with solutions of fluorescent dyes or by addition of inorganic fluorescent materials to the adsorbent. When such a thin-layer plate is viewed under ultraviolet light the material can be recognized as dark spots on a fluorescent background.

Adsorbents for thin-layer chromatography having fluorescent indicators added are available commercially. They are usually distinguished by the letter F, following the type of adsorbent.

The addition of this inorganic fluorescent dye to the adsorbent is extremely simple and does not interfere with separation or extraction of the organic materials.

Applications of this technique can be found in the books of Stahl, and Randerath[1, 2]

C. Color Forming Reagents

Very many compounds are not characterized by the possession of color, strong absorption in the ultraviolet, or fluorescence. They have to be rendered visible by special detection reagents which are usually sprayed on the developed chromatogram. The reagent is chosen to give color or some other means of detection.

Many of the reagents in use are applicable to both paper and thin-layer chromatography. Other reagents of special applications can be found in Feigls' book which contains a vast number of reagents that are suitable for chromatography.[3] In addition, highly aggressive reagents can be used with the inorganic layers of thin-layer chromatography.

Some of the more recognized spray reagents are listed in the books by Stahl, and the book on paper chromatography by Hais and Macek.[1, 4] These books cover over a hundred reagents including their preparation and use.

Care should be taken when spraying that too much reagent is not used. This can cause the spots to diffuse or wash off and the chromatogram would be wasted. Because the color-formed may fade after a short time, the spot should be ringed or otherwise marked as soon as possible.

A number of mechanical devices are available with which a chromatogram can be sprayed. They range from devices which may be made from a test tube to a propellent-gas sprayer with a changeable glass flask.

It is important to spray the reagent solution on to the chromatogram in the finest possible dispersion i.e., in the form of an aerosol. Any device which can do this can be used. The commercially available devices, however, will usually give better spraying conditions than the homemade types.

D. Charring:

One method in use with thin-layer chromatography uses a technique which converts organic compounds to charcoal for visual detection. The plate is sprayed with sulfuric acid and heated to a high temperature with the organic materials appearing as black spots on the chromatogram.

This particular method and others which use such aggressive reagents can be carried out only on the more stable inorganic layers used in thin-layer chromatography.

E. Other Methods:

There are other methods for the detection of spots. One such method utilizes the inhibition or acceleration of growth of suitable biological organisms. However, these methods have little application for air pollutants and will not be covered here. These methods are covered in the texts included in the references at the end of this outline.

III DOCUMENTATION

Information gained from chromatographic procedures must be recorded in some manner. Properly recorded it can be used for comparison to establish the identity of an unknown. The amount of information recorded can vary with need. However, the conditions of development, type of adsorbent or paper and other information to identify the chromatogram should be recorded. The R values of standard and unknowns and their colors should also be recorded. Because of the large amount of information to be recorded it is recommended that a printed form be used in order to shorten the time.

Paper chromatography lends itself to documentation more easily than any other type of solution chromatography. After the development of the paper chromatogram it can be stored and used, by itself, as a record. However, if the spots tend to fade or in some manner lose their location it too must be documented.

Thin-layer chromatograms are more easily damaged and therefore are not easily stored. The glass plates, used as a support with some chromatograms, can be reused and it is advised to find other methods of documentation.

The following methods may be applicable only to one type of chromatography or may not even be needed as with paper chromatography. The investigator is advised to consult the reference books on the subject for more detailed information on the various methods of documentation.

A Drawing:

The simplest method of documentation is to draw the chromatogram by hand. It is not sufficient to record the location of the spots in a chromatogram merely by giving the Rf values as can be done with paper chromatography. According to Stahl:

> "....the Rf values are regarded rather as guides which give the relative position of the spots within a chromatogram. It is, therefore, necessary to make very detailed notes of all experimental conditions when carrying out T.L.C."

When drawing the plate a printed form should be made a part of the drawing. This form should include all the pertinent information concerning the conditions under which the chromatogram was developed. With this information the chromatogram can later serve as a reference to the separation under the preset conditions.

Drawing the plate will not reproduce the colors or fluorescence and these are very important for future identification of the separated materials. Therefore, some method of documentation must be found for methods other than routine.

PHOTOGRAPHY:

This method of documentation has the advantage of being permanent, giving good reproduction of colors and fluorescence, being easy to store and relatively fast. The method requires more investment in equipment than most methods but gives the most favorable results.

Photographic recordings have been used, for both paper and thin-layer chromatograms. The camera can be used in both daylight, or with a viewing device for photographs in the ultra-violet.

Several of the reference texts mentioned go into much more detail on this subject and it is recommended that if more information is necessary that these references be consulted.

PRESERVATIVES:

Probably the most widely used method is a technique that permits intact removal of the thin-layer adsorbent. Special preserving materials for this purpose are marketed by several commercial suppliers. These preservatives are sprayed on the layer, allowed to dry, and the coating can then be peeled off intact.

There are some disadvantages with this procedure however. With some adsorbents being harder to remove than others. Colored spots, in time, may fade thereby reducing the value of this type of record. Considerable technique is necessary on the part of the analyst to remove the complete layer.

The cost is less expensive than some of the previous methods and storage is no problem. If a data sheet is stored with the preserviced layer, a complete record of the plate is obtained.

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SECTION VII

SPECIAL TECHNIQUES

SPECIAL TECHNIQUES

J. D. Pfaff*

I INTRODUCTION

The purpose of this outline is to cover the remaining techniques not covered in previous outlines. These methods are not used as widely as the methods already covered. Several of the techniques covered here are merely modifications which will have only specialized application. Familarization with these techniques is necessary in order to obtain a complete picture of the uses and possibilities of the various types of chromatography.

In general the methods considered here were developed to have specific applications in the development of methods in research. They are usually more difficult to use, require more technique, and are necessary only for very small quantities and concentrations. The simpler a method is kept the more reproducible and less time consuming it becomes.

These methods, with certain exceptions tend to become more expensive than the standardized procedures. This however, in the case of precoated plates, is overcome by the greater advantages involved in using these types of plates.

II ELECTROPHORESIS

Thin-layer electrophoresis corresponds closely to paper electrophoresis in method. A thin-layer prepared in the usual way and soaked with a suitable buffer solution serves as the support. Strips of paper connect the layer with the electrode chambers, which are filled with an electrolyte solution. Electrophoresis is commenced by applying a D.C. voltage.

Some of the earliest work on electrophoretic principles was done by Berraz in Argentina in 1943. Paper electrophoresis is still not used as widely as the simpler types of chromatography. However, it has found considerable use in the separation of high molecular weight substances. Little application to the field of air pollution has been noted. However one recent paper describes a technique used for the separations of polynuclear phenols obtained from air samples.(2)

Most electrophoretic procedures fall into two catagories, i.e. high voltage and low voltage. The main difference being the time of separation. When a low potential is applied across a paper which has been completely saturated with a suitable buffer the spots will migrate at a very slow rate. If the separation is not of large degree, diffusion over the long time period will negate the entire separation. For this reason a high potential is used to decrease the separation time and give the material less time to diffuse. Other variables which can effect the separation include pH and temperature. With the increase in potential gradient, the migration rate of ions increases linearly, but the heat generated increases quadratically. Thus, when the potential gradient is increased from 5V/cm to 100V/cm, the zones will migrate about 20 times as fast, but the amount of heat generated in the strip will increase 400 fold. Therefore, with high voltage electrophoresis cooling is a necessity. This is usually accomplished by the use of organic liquid heat exchangers. The type of apparatus used in electrophoresis is shown in Figure 1 and Figure 2. The equipment in general is about the same for

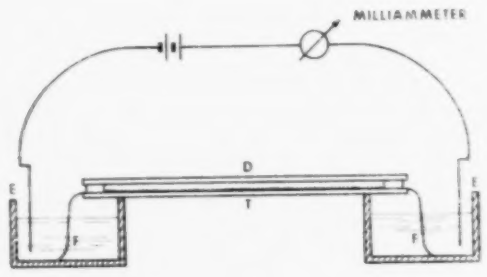


Figure 1

*Chemist, Water Hygiene, Training Institute

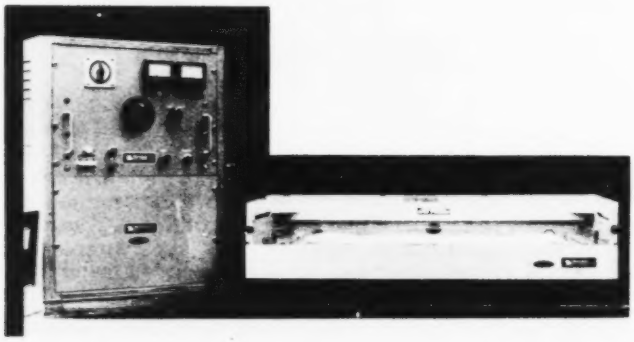


Figure 2

paper or thin-layer electrophoresis. Figure 1 is an early piece of equipment while Figure 2 shows equipment available today. Note that in Figure 2 the equipment is capable of being cooled by using a liquid to circulate through the equipment.

III TWO DIMENSIONAL CHROMATOGRAPHY

Two dimensional chromatrography is an extension of the normal development of paper and thin-layer chromatography. The developers of paper chromatography (Donsden, Gorden and Martin) saw the advantages of this type separation. The two dimensional technique is a valuable tool for complex mixtures.

Separation using this technique is first accomplished in one direction, then the plate is rotated through 90° after which the separation is again allowed to proceed.

Two dimensional chromatography may be divided into two groups according to the systems used to accomplish the separations. The first method is the development using identical developers in both directions. The advantage here is that the area of separation is doubled in effect without the disadvantage of having very outsized plates or papers. The second method is the development in the first direction with one solvent then allowing the chromatogram to dry and developing in the second direction using a different solvent. The advantage of two different solvent systems and their different separatory powers can be seen along with the extension of the area of separation. The separation of the very complex mixtures of the benzene extracted portion of an air sample can be more easily achieved using the technique of two dimensional chromatography. Several papers using this technique for the separation of air pollutants have been published.[2, 3]

As with all other types of chromatography, the important aspect of identifying the separated spots must still be considered. In most, instances, the spots from the chromatograms are eluted and their spectra obtained using ultraviolet or fluorescent techniques.

Two dimensional chromatography has the same disadvantages as the normal types of chromatography. It must be stressed that the factors which effect the reproducibility of one dimensional methods have an even greater effect on two dimensional methods. Thus, for instance, the conditions of the intermediate drying must never be altered. Standards are usually run in both directions in order to assure easy location of the wanted materials. These standards will be displaced an equal amount as the separating materials by any deviation in the standard operating procedure. This will help to minimize changes, and give more useful results.

IV CIRCULAR CHROMATOGRAPHY

This method is one of the oldest forms of chromatography having been used about 100 years ago. In radial or circular chromatography the support (i.e. plate or paper) is

held horizontally (Figure 3). The solvent is supplied to the center of the support. This can be done using a wick to feed the solvent from the supply to the support material. The system can be kept enclosed for the entire run thus providing a saturated atmosphere.

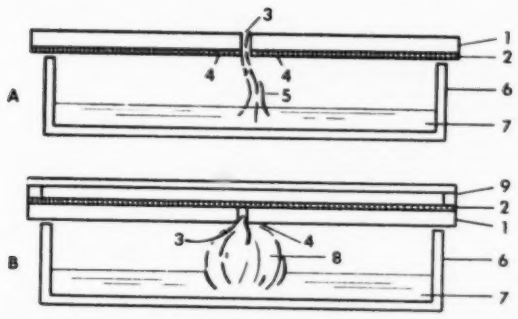


Figure 3

Originally, only one sample was applied to the center of the support. Now however samples are applied to the circumference of a circle about 1-3 cm. in diameter and the solvent system supplied to the center of the circle. The support can be modified in order to prevent the samples from running together. Paper is available (Figure 4) which has radial slots cut into the paper.

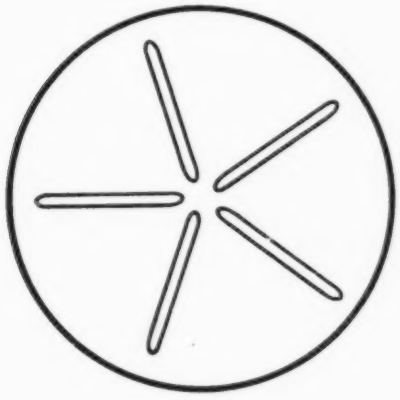


Figure 4

Circular chromatography is usually much faster in development than the ascending or decending methods of development. One disadvantage is that fewer spots can be run at one time. Also as the spots migrate higher they tend to diffuse more readily and therefore become less concentrated.

V MODIFICATION

Under this section will be considered modifications of existing equipment. Modifications which were designed to prevent or cause specific changes.

A The first such modification to be considered is what is termed the wedged-tip-technique. As many other techniques this slight change was developed for paper chromatography. This technique uses wedge shapes cut into the absorbent as shown in Figure 5. The vertical lines emanating from the apex of the pentagon shaped wedge limits the path of development. Due to this shaping of the paper or thin-layer the substances being separated are forced to assume an almost band like path. These long narrow bands do not overlap if the differences in the migration rates are small as they can be in the normal types of separation. Papers cut in this wedge shaped are commercially available as well as a template that can be used to cut in the wedges on a thin-layer chromatogram.

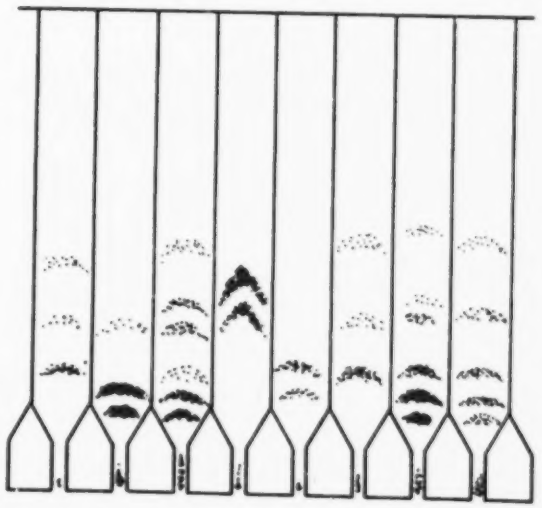


Figure 5

B Another variation designed for the same purpose is known as scoring of the plate. This modification finds use primarily in thin-layer chromatography. Although the wedges are omitted the vertical lines are scored or cut into the adsorbent. Scoring has the same use as the wedge-tip-technique but is less troublesome to do.

C The degree of freedom in the use of adsorbents is one of its greatest advantages of thin-layer chromatography. Not only is there a wide choice of adsorbents capable of being used but there is also the ability to mix the adsorbents with each other.

In 1964, a new type of T. L. C., called Gradient-Layer chromatography was introduced by Stahl. In this procedure, instead of coating a plate with only one absorbent, two different slurries are made up and are applied simultaneously in such a way that the layer is made up of a gradient mixture of both adsorbents. For example, by using aluminum oxide and silica gel, a pH gradient is obtained across the plate. Because of the great variety of adsorbents which are available, a considerable number of combinations are possible. One type of spreader for this gradient technique is shown in Figure 8.

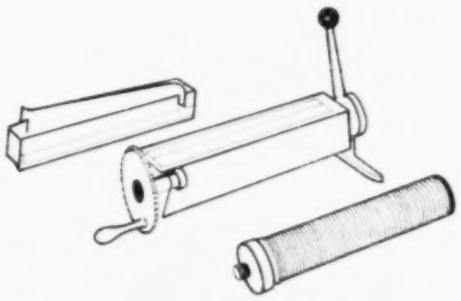
For some purpose it may be desirable to produce two or more parallel bands of different adsorbent layers on the same plate. If these bands are arranged horizontally sample would travel through each band successively and fractions of the sample which might not separate on one adsorbent would continue as a mixture to the second or third adsorbent where resolution could occur. This modification can be accomplished using a type of spreader as shown in Figure 9.

D Sandwich Chambers

A unique system has been devised for the development of a single chromatogram. It is referred to as a "sandwich" chamber, a name which is quite descriptive of the setup (Camag "Sandwich" Chamber). An ordinary TLC plate is spotted and allowed to dry. A strip of the adsorbent layer 8 - 10 mm wide is scraped off the side and top edges of the plate. A (see Figure 6) shaped cardboard spacer is placed over this strip. Then another glass plate of the same dimensions is placed over the spotted plate and the two are clamped together. Then the open end of the assembly is placed in a shallow trough containing the solvent. The glass plate forms the front wall of the chamber and the chromatoplate itself acts as the back wall. (See Figure 7) No special developing tank is needed and - less solvent is used for development, thereby lessening equilibration time.

Another variation of the sandwich chamber consists of a special glass front plate which was glass supporting rails fused to its sides (Brinkmann Stainless Steel Sandwich Developing Tank). This eliminates the need for cardboard spacers. The

Individual parts of the applicator



Assembled applicator

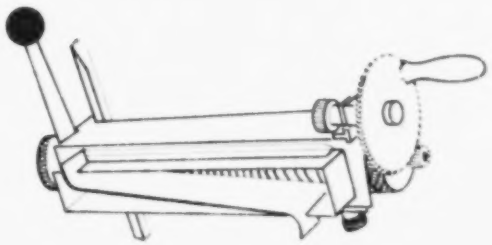


Figure 6

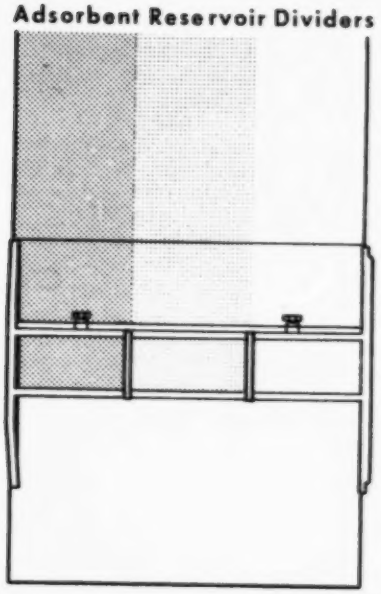


Figure 7

chromatoplate itself again forms the back wall. Development is carried out in the same manner as described above.

Mention might also be made of prepared sandwich chambers (Analtech "Unichamber"). The same principles apply, but the chamber is preformed, and the sample is spotted on the finished sandwich arrangement. (17)

VI PRECOATED PLATES

In recent years considerable effort has been directed toward simplification of the thin-layer chromatographic techniques by the introduction of the precoated, ready to use layers. When first introduced these plates had problems with the layers falling off and were in general less desirable to use than home made plates. The layer also varied from plate to plate and reproducibility was poor. As more manufacturers offered these premade plates and new techniques were developed for their production their reliability became greater and greater. Now these plates are generally accepted by most users of thin-layer chromatography.

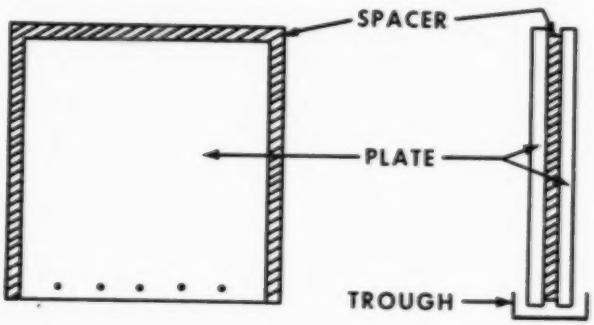


Figure 8

Figure 9

The unit cost of precoated layers is not much higher than local coating and do offer numerous advantages in regards to quality, convenience, and time saved. The reproducibility for most of these plates is generally better now, than those made in an individuals own lab. Usually as the number of precoated plates purchased rises the cost lowers.

These precoated layers are also available coated on thin-films of plastic. This type of chromatogram is more easily stored and easily cut to shape. A significant amount of work has been done cutting separated spots from this type of chromatogram in order to obtain their fluorometric spectra directly.

The advantages in using these precoated chromatograms (both glass and plastic supported) are many and have been mentioned. However, if a person is doing research it is still advisable to produce his own plates inorder that all the various techniques of chromatography are at his command. When a standardized procedure is being done the precoated chromatograms are most useful.

One other type of chromatogram should be mentioned here. This is the chromatotube, which is a round glass tube which is coated on the inside with a thin-layer of adsorbent. The tube can be closed at one end and forms its own developing tank. The advantage of these tubes are their reproducibility which is very good. This is because the chamber volume and shape is standardized and developing chamber saturation is more reproducible.

The disadvantage would be the removal of the separated materials for other uses. This would be difficult just as removal from column chromatography is difficult.

(Precoated chromatograms are available from a number of manufacturers and can be purchased in a great variety of coatings)

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SECTION VIII

QUALITATIVE AND QUANTITATIVE CHROMATOGRAPHY

QUALITATIVE AND QUANTITATIVE CHROMATOGRAPHY

I INTRODUCTION

In order for any method to be classed an analytical it must be able to determine some material either qualitatively or quantitatively. Chromatographic methods are no exception. With the chromatographic methods we are considering, the separated material must first be located. After location it is then possible to determine what the material is and how much is present. The methods of location have been discussed in the outline on spot detection and documentation. It should be stressed here that it is imperative that correct and complete documentation be achieved. This is especially important in the case of thin-layer chromatography where storage is more of a problem.

II QUALITATIVE EVALUATION

After location of the spot qualitative analysis can be performed by calculating the R_f value. The calculation can be simplified by the use of a measuring template. This device is available from many supply companies. These templates are usually measured from an origin to fifteen centimeters. The chromatogram is often stopped after it has run 10 c m. If this is the developing distance, then ten times the R_f value can be read directly from some measuring device or a template.

The R_f value is given by the ratio of the distances traveled by the spot to the distance traveled by the solvent. The distance traveled by the spot is usually determined by measuring to the center of the spot. However, when reading the current literature values are sometimes found using the distance measured to the leading edge. This is done trying to eliminate the effects of tailing. Another value found in the literature is the R_x value. This value is referred to as the R standard value (ie Rst) and is defined as the R_f value of a spot divided by the R_f of a chosen standard. This value usually expresses the values when a series of plates has been used to evaluate the separation of a large group of materials.

The R_f value for any compound is found to be most reliable when it is determined from the mean of a series of independent chromatograms. In a previous outline it was pointed out that many variables can cause a deviation of a compounds R_f value. Therefore, it is extremely important to keep every step involved in the development of a chromatogram as constant as possible. It is recommended that before any chromatographic technique is used for either qualitative or quantitative work that the analyst read the section on reproducibility in one of the reference works available. It would also be wise to evaluate the technique involved by running standards and checking their reproducibility in the chosen method.

When considering the various methods for developing a chromatogram, i.e. ascending, decending and horizontal flow, the analyst should not expect the R_f values for a compound to compare. Even if all other factors remain constant on an ascending strip, gravitational force reduces the amount of solvent and hence, reduces the R_f value. Conversely, on a descending strip, gravity increases the amount of solvent and increases the R_f value.

Since all variations which effect the R_f value are significant, each R_f value reported in the literature should include such pertinent, information as the solvent system, support material, equilibration time, temperature, direction of development and development height along with any special conditions.

The various R values found in use in most major reference works are listed in Table I. Their definitions may vary slightly from author to author but are generally defined as listed.

When comparing substances of known structure it is often possible to make qualitative predictions using the ratio of the respective partition constant (K') values by using certain solubility rules. A larger K' value corresponds to a larger R_f value or a smaller retentions volume. In this way, useful qualitative predictions can be made regarding the chromatographic separation. From certain mathematical derivations it has been shown that the Rm value is a linear function of the contributing groups which make up the molecule. Thus the Rm value can be said to show a relationship between structure and the partition constant of a compound.

III QUANTITATIVE EVALUATION

Quantitative evaluation of a chromatogram is much more than the qualitative evaluation.

Table I.

R	=	the fractions of solute molecules in the solvent at any one time.
R_R	=	Radial distance to zone/Radial distance to solvent front (circular chromatography).
R_f	=	Distance traveled by leading front of solute zone/distance traveled by solvent front.
R_x	=	Distance traveled by the solute/ distance traveled by reference substance.
Rst	=	same as Rx
hRf	=	$R_f \cdot 100$
Rm	=	log ($^1/R_f$-1) shows relation between structure and the equilibrium constant (K^1) of a material.

All the precautions mentioned under qualitative evaluation are even more important when quantitative work is to be done. Methods which are specific enough for the determination of a single, or limited number of substances in a complex mixture are still few in number. This is why separation methods are gaining more popularity in air pollution work. These separation methods first separate the complex samples and then allow the quantitative determination to be carried out by some method which is not sufficiently selective. The various types of chromatography are particularly important for this, because of their relative simplicity and minimal apparatus requirements and the quantity of sample needed.

Thin-layer chromatograms are capable of being quantitatively determined just like paper chromatograms. Randerath says that an error of 5 to 10% is to be expected when quantitation of a thin-layer chromatogrone is carried out.[1] However, other methods using chromatography for the separation of hydrocarbons recover only 50 to 80%, depending on the quantitative method.[2]

The determination after preliminary separation on the chromatogram can be performed either after elution or directly on the adsorbent. The various ways of carrying out these determinations will be discussed here. However, if more detail is desired, the reference works of chromatography should be consulted.

A Determination Without Extraction

1 Comparison of Spot Size

This method is about the first attempt to quantitate chromatography and is still in use today. In this procedure the test material is applied in the usual manner and the appropriate standards applied next to it in several concentrations. After development and detection is complete, the size of the spot area is evaluated and compared to the dilution series of the standard. This method is used with paper chromatography and quite often gives good results. However, with thin-layer chromatography less accurate results are quite frequently obtained. Measurement by visual comparison of spot size is at best a semiquantitative method which can involve error of about 20 to 40%. This method as well as any visual or color measuring method precludes that the spot have color or can be stained. When a spot is stained there is the possibility of incomplete staining or interference from the stain itself. However, the main advantages of visual comparison is speed and simplicity.

2 Measurement by Photo-densitometry

Measurement by photometry is an attempt to achieve a curve or an absorbance curve. After suitable color development the chromatogram is drawn across a slit in front of a photocell and the transmittance readings are plotted on a graph. The area between the peaks can be measured by planimetry or weighing and comparing with a calibration curve. If the curve expresses absorbance and the absorbance obeys Beer's law, the area under the peak is proportional to the amount of the substance in the spot. This same technique is possible using visible, ultra-violet or fluorescence techniques.

The densitometric method will lead to rapid results, if the background does not interfere very much and if there exists, over a certain range, a reproducible relationship between spot area and quantity of substance. Errors of from 5 to 10% are to be expected using this technique.

B Determination using Extraction

Methods in this group have had the largest degree of application toward pollutants found

in the atmosphere. Methods, after extraction, are in the literature using fluorescence, phosphorescence, ultra-violet and visible spectrophotometry to identify the separated materials. In a determination after elution the error may be as low as 1-2% depending primarily on the completeness of extraction.

When using elution methods the first step, in the washing off (eluting) of the compounds is the most important. This step must be carried out quantitatively and is usually expected that approximately 95% of the spotted material can be re-extracted. In order to elute a substance from a chromatogram, its position must first be ascertained. Any of the methods of location can be used, but care must be taken to insure that no change, in the material, not expected take place.

Depending on the type of photometry used varying amounts of material must be present. For example, it was found in the analysis of polynuclears that amounts of 10-20 μg must be present in order to use spectrophotometry while fluorescence needed 0.2 to 0.4 μg for a successful determination.

IV SUMMARY

Chromatography can be both qualitative and quantitative in nature. Qualitative chromatography uses the R_f and other R values to determine the identity of a compound. However, certain drawbacks exist such as the necessity of standards with each run, exactness in technique and the location of the separated material. Quantitation poses more problems and one can expect errors of 1 to 50% in the results. However, the use of chromatography is growing and more work is appearing in the literature. Speed and simplicity are two advantages of chromatography which cannot be overlooked when a material must be both separated and identified.

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SECTION IX

CHROMATOGRAPHY OF PESTICIDES

ANALYSIS OF PESTICIDES IN THE ENVIRONMENT

Steven N. Dereniuk[1]

Since the end of World War II a large number of organic chemicals have been developed for the control of a variety of pests.

These are used on grain, fruit and vegetable crops, pastures, forests, waters, farm animals, barns, warehouses, houses, gardens and lawns. Chemicals are also used to defoliate, to control brush, to prevent postharvest decay, to treat seed and fruit settings, to treat lumber, to dessicate, to repel, etc.

In the USA losses in agriculture caused by pests are estimated to be in excess of 15 billion dollars annually during growing, processing, storage and marketing. Without pesticides the quality, quantity and cheapness of food we now have would hardly be possible. Besides causing high food and fiber losses pests also spread disease and damage our possessions. When we say pests we mean insects, rodents, weeds, pest birds and fish, bacteria, fungi, nematodes, protozoa, etc.

We now use in the USA in excess of 750 million pounds of pesticides annually in agricultural and non agricultural endeavor. This is applied with land and aerial equipment.

Although pesticides bring about great benefits in increased food and fiber supplies and in the control of many diseases these benefits do not come without ill effects. Pesticides improperly used can be harmful to man and animals causing illness and even death. Extreme care should be exercised in the application, handling, storage and shipment of pesticides. Food, feed, drugs, clothing, toys and other materials should not be stored or shipped with pesticides lest these items become contaminated with pesticides.

Pesticide residues are now found in food, water, soil and the atmosphere. Waters accumulate pesticides from direct application, drift, run off from treated soils and dumping from industrial plants; soils from direct application and drift; the atmosphere from direct application, dust from treated soils, volatilization and burning of spent containers; livestock from direct application, treated barns and pens, feed and pasture; wildlife via the food chain; man from food, from working with pesticides, from living in localities where pesticides are heavily used and from items accidently contaminated with pesticides.

In a recent FDA survey involving 100,000 food samples, about half were found to contain at least one pesticide. Approximately 3% of the samples exceeded legal residue tolerances or in the absence of legal tolerance exceeded guidelines for tolerances.

Protection against pesticide residues in foods and milk is provided for by the Federal Food, Drug and Cosmetics Act of 1938 and the 1954 Miller Amendment to Food, Drug and Cosmetics Act. States also have laws regulating pesticides.

The rapid accumulation of pesticide residue data came only after the development of the techniques of gas liquid chromatography, thin layer chromatography and paper chromatography.

In the organic class of pesticides are the nonionic chlorinated hydrocarbons, the ionic chlorinated hydrocarbons (herbicides), organic phosphates, carbamates, thiocyanates, dithiocarbamates, dinitrocresols, dinitrophenols, petroleum oils and ethyl and methyl halides and other organics; in the inorganic group are compounds of mercury, sulfur, arsenic, phosphorus, zinc, lead, copper, bismuth, fluorine, chlorine, boron, etc.; from the botanicals come pyrethrin, rotenone, ryania, sabadella and nicotine. There are other organics, inorganics and botanicals.

One problem in pesticides residues studies is the low concentrations at which pesticides chemicals are found in the environment. In foods, feeds, plant and animal tissues and soils pesticides concentrations may be at parts per million or less. In the aquatic environment because flora and fauna can concentrate pesticides in their tissues concentration levels at parts per trillion can be significant.

These small amounts of residue have to be extracted quantitatively, freed from interfering materials and often concentrated many fold in order to be suitable for detection by gas chromatography methods.

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Extraction is carried out with polar, nonpolar or a mixture of polar and nonpolar solvents. The choice of solvent is determined by the nature of the sample and the polarities of the pesticides.

The first extraction of a biological sample results in a great reduction in mass as most biological materials have a high water content which does not pass into the organic phase. Also cellulose, proteins, carbohydrates and related compounds are only slightly soluble in organic solvents. However lipids, pigments and many other compounds are extracted by organic solvents. The extract thus has to be carried through a cleanup step to remove these interfering materials. Several procedures are available for cleanup. The most often used are partition, chromatography and chemical treatment.

In cleanup by partition two immiscible solvents in a separatory funnel are used; a nonpolar solvent (hexane or petroleum ether) and a polar solvent such as acetonitrile. The fatty materials are carried into the nonpolar phase and the pesticides into the polar phase. This procedure removes oils and fats but not polar lipids, pigments and other materials. To complete the cleanup the residue is passed through a column of florisil, silicic acid, alumina oxide or some other adsorbent. An ideal adsorbent is one with a high affinity for interfering materials and low affinity for pesticides. Thin layer chromatography can also be used for cleanup. Lipides can also be removed by saponfication with alchoholic potassium hydroxide or treatment with concentrated sulfuric acid impregnated on a solid support. This latter treatment is not as applicable as partition and sorption as many pesticides are destroyed by acids and alkalies.

After extraction, cleanup and concentration the pesticide residues are measured with a gas chromatography employing the necessary detector or detectors. The most sensitive detector is the electron capture detector which can measure some pesticides at 10 pg level.

The fate of pesticides in the environment is a function of many interrelating factors. Their breakdown is affected by light, temperature, moisture, soil, nature of the chemical, type of application, etc. They may be metabolized by plants, animals and microorganisms. In some cases the breakdown products are more toxic than the parent compound. DDT is dehydrochlorinated to DDE and then oxidized to DDA. Aldrin is oxidized to dieldrin by plant and animal tissues and soils. Heptachlor is converted heptachlor epoxide while parathion and other sulfur containing organic phosphates are oxidized to the oxons. Some of the oxons are more toxic than the parent compounds. Parathion is converted to the more toxic paraoxon by plant enzymes. In animals parathion is metabolized to p-nitrophenol and p-aminophenol. The breakdown products of many of the pesticides are not known. Thus the absence of a pesticide does not necessarily rule out the absence of a toxic compound.

In pesticide residue analysis the following weight and volume terminology is used:

Unit	Abbreviation	Fraction of a gram
gram	g	1
microgram	µg	10^{-6}
nanogram	ng	10^{-9}
picogram	pg	10^{-12}

Thus 1 µg per gram is one part per million (1 ppm), 1 ng per gram is one part per billion while 1 pg per gram is one part per trillion.

The volume measurement for GC injection is the microliter (µl) or the lambda (λ) which is 0.001 ml.

For the laboratory exercise a milk sample extraction procedure is outlined.

DETERMINATION OF NONIONIC CHLORINATED ORGANIC PESTICIDE RESIDUES IN MILK

EQUIPMENT

Centrifuge, explosion proof

Centrifuge bottles, 500 ml

Kaderna-Danish concentrator, 500 ml with 3 ball Snyder column

Graduated tubes for Kaderna-Danish, 4 and 10 ml with 19/22 joints

Volumetric flasks, glass stoppered, 10, 25, 50 and 100 ml

Erlenmeyer flask, glass stoppered, 125, 250 and 500 ml

Separatory funnels, Teflon stopcocks, 125 and 500 ml and 1 and 2 l.

Graduated cylinders, 100 and 500 ml

Funnels

Transfer tube

Vials with Teflon lined screw caps

Columns, glass, 22 mm i.d. × 300 mm, Teflon stopcocks and course porosity discs

Steam bath

Double strength glass plates, 8 × 8"

Capillary pipettes, 5 µl

Spotting template

Dessicator for plates

Drying oven

Developing tanks

Box with ultra violet light

REAGENTS

Alcohol, ethyl or methyl, reagent grade

Petroleum ether, distilled in glass

Hexane, distilled in glass

Acetonitrile, distilled in glass (acetonitrile is extremely toxic; use with caution)

n-heptane, reagent grade

Acetone, reagent grade

Potassium oxalate, reagent grade

Sodium sulfate, anhy., granular, reagent grade

Sodium chloride, reagent grade

Florisil, activated

Silver nitrate

Rhodamine B, spirit soluble

Nitric acid, 70-71%, reagent grade

Aluminum oxide (Merck)

Pesticides

REAGENT MIXTURES

Acetonitrile saturated with petroleum ether (approximately 15 ml hexane per 100 ml acetonitrile)

Eluting mixture (6+94). 6% (V/V) ethyl ether in petroleum ether

Eluting mixture (15+85). 15% (V/V) ethyl ether in petroleum ether

Mixed ethers (1+1). Equal volumes of ethyl ether and petroleum ether

Sodium chloride, saturated aqueous solution

Sodium chloride, 2% aqueous solution

Acetone in n-heptane. 2% acetone in n-heptane

Nitric acid, 0.03N. 1 ml of nitric acid in 500 ml of distilled water

Ethyl ether in hexane. 1% ethyl ether in hexane

Rhodamine B. 0.01% rhodamine B in 95% ethanol

PROCEDURE

To 100 ml of fluid milk in a centrifuge bottle add 100 ml ethyl alcohol and 1 g K oxalate and mix.

Add 50 ml ethyl ether and shake vigorously for 1 min.

Add 50 ml petroleum ether and shake vigorously for 1 min.

Centrifuge for 5 min. at about 1500 rpm.

Transfer solvent with transfer tube into a 1 l. separatory funnel containing 500-600 ml H_2O.

Extract milk two more times with 50 ml portions of mixed ethers. Transfer solvent to original 1 l. separatory funnel.

Agitate separatory funnel gently but thoroughly. Let layers separate; discard H_2O.

Wash solvent twice more with 100 ml portions of H_2O; discard H_2O each time.

Pass ether through 2" deep column of anhydrous Na_2SO_4. Collect solvent in beaker.

Wash column with small portions of petroleum ether.

Evaporate combined solvents to obtain fat weight.

Transfer fat to a 125 ml separatory funnel with small portions of petroleum ether. Hold total volume of fat and ether at 15 ml.

Add 30 ml acetonitrile saturated with petroleum ether, shake vigorously for 1 min., let layers separate and transfer acetonitrile into a 1 l. separatory funnel containing 700 ml of 2% NaCl solution and 100 ml petroleum ether.

Extract petroleum ether 3 more times with 30 ml portions of acetonitrile saturated with petroleum ether. Add extracts to 1 l. separatory funnel.

Stopper 1 l. separator, invert and vent off pressure. Mix by tumbling separator for 15 sec.

After layers separate transfer aqueous layer to second 1 l. separator.

Wash aqueous layer with 100 ml of petroleum ether.

Discard aqueous layer

Combine petroleum ether fractions and wash twice with 100 ml portions of H_2O. If emulsion forms add about 5 ml of saturated NaCl solution to break emulsion. Discard washings.

Pass petroleum ether through a 2" deep column of anhy. Na_2SO_4. Wash separator and column 3 times with 10 ml portions of petroleum ether; collect ether in a Kaderna-Danish concentrator.

Evaporate to about 10 ml.

Prepare a florisil column. To a 22mm i.d. × 300 mm glass column add enough activated florisil to give a 4" column after settling. Top with about 1/2" of anhy. Na_2SO_4. Place Kaderna-Danish concentrator under column.

Transfer extract unto column. Rinse container with two 5 ml portions of petroleum ether and add to column. Rinse column with small portions of petroleum ether.

Elute at about 5 ml per minute with 200 ml of 6% eluting mixture. Change receivers and elute with 200 ml of 15% eluting mixture.

Concentrate each eluate on a steam bath to 1 ml or less. The concentrate is ready for TLC.

If further cleanup is necessary, repeat cleanup using a new florisil column.

PREPARATION OF PLATES

Add 40 g Al_2O_3 to a 500 ml centrifuge bottle. Record total weight.

Add 80 ml 0.03N HNO_3, shake well and centrifuge 1-2 min. at about 1200 rpm.

Decant supernatant liquid, add 80 ml distilled H_2O, break up cake with glass rod and centrifuge as before. Decant supernatant.

Wash twice more with 80 ml portions of H_2O.

Add 10 ml 1.0% $AgNo_3$ and enough H_2O to make Al_2O_3 and liquid weight total 120 g.

Break up cake and shake well to resuspend Al_2O_3. Place in applicator and prepare plates.

Dry plates on mounting board for about 15 min.

Place plates in vertical position in drying rack and dry at 100°C for 30 min.

Remove plates and store in dessicator.

Prepare a slurry of 40 g of Al_2O_3 in 80 ml of H_2O.

Place in applicator and prepare plates.

Dry plates on mounting board for about 15 min., place in vertical position in drying rack and dry at 100°C for 30 min.

Store in a dessicator.

Prepare individual solutions of DDT, DDE, TDE, heptachlor, heptachlor epoxide, aldrin and dieldrin at a conc. of

1.0 mg/ml in hexane. Prepare a working solution of each pesticide containing 0.1 mg/ml in hexane. Prepare a solution in hexane containing 0.1 mg/ml each of DDT, DDE and TDE. Prepare a solution in hexane containing 0.1 mg/ml each of heptachlor, heptachlor epoxide, aldrin and dieldrin.

Take a plate coated with Al_2O_3 and impregnated with $AgNO_3$ and scrape 0.25" of coating from each side. Score line across top of plate 5.5" from bottom edge.

Make a mark at each side of the layer 1" from the bottom of the plate. Place a spotting template across the two marks and spot the working pesticide solutions and the sample extracts.

Apply 5 µl pesticides solution; apply 1/2 the contents of the capillary, let spot dry and apply remainder of the contents. Rinse capillary twice with hexane and apply next solution. Apply 5-20 µl of the sample extract.

Line developing tank with filter paper sheet, add 100 ml of 2% acetone in n-heptane, cover tank and let it equibrate for 15 min.

Place spotted plate in tank, replace cover and develop until solvent layer just reaches the top line on plate.

Remove plate and dry in hood for 5 min.

Place plate in ultra violet box for 10 min.

Remove plate and note spots. Measure Rf value of the knowns and unknowns. Record data.

Spot plate coated only with Al_2O_3.

DEVELOP PLATE

Dry developed plate and in hood spray plate with Rhodamine B solution.

Place plate under ultra violet light and mark spots.

Insert a tuft of glass wool into a medicine dropper. Connect tapered end of medicine drop to vacuum line. Collect desired spot of adsorbent.

Place medicine dropper over small graduated tube and elute pesticide with 5 ml of 4:1 hexane-ethyl ether mixture.

Concentrate elute on steam bath to 0.5 ml of less.

The concentrate is suitable for infrared spectrophotometry and gas chromatography.

NOTES ON PROCEDURE

Chemicals should be reagent grade.

Acetonitrile is purified as follows: to one gallon of acetonitrile in a glass still add 1 ml of 85% H_3PO_4, 30 g P_2O_5 and boiling chips and distill at 81-82°C. Vapors from impure acetonitrile will turn moistened litmus paper blue. Acetonitrile is very toxic.

The florisil should be a PR grade, activated, 60-100 mesh, calcined at 1250°F for 3 hr. (Floridin Co., 300 Penn Center Blvd., Pittsburgh, Pennsylvania). Store in amber glass bottles with glass stopper or metal screw cap. Prior to use heat 5 hr. at 130°C and store at 130°C. If stored in dessicator at room temperature reheat at 130°C after 2 days prior to use.

Properly cleaned up samples can be analyzed by TLC.

Aluminum oxide is superior to silica gel for TLC of chlorinated organic pesticides (nonionic).

TLC can be used for cleanup of samples for infrared spectrophotometry, gas chromatography, etc.

The procedure given for determining chlorinated organic pesticides in milk is applicable with some modification to plant and animal tissues

and to foods and feeds. Soils are complex and present problems in extraction; benzene is a good solvent for extracting chlorinated organic pesticides from soil.

Water can be satisfactorily extracted with hexane and other solvents. Finished water requires no cleanup. A one liter sample can be extracted with 10 ml of hexane and the hexane extract concentrated in a Kaderno-Danish concentrator. Raw water extracts may require considerable cleanup.

A study of pesticides in the atmosphere involves running determinations on extracts of (1) rainwater samples (2) dust samples (3) filter from air sample collectors and (4) organic solvents used in air scrubbing operations.

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SECTION X

CHROMATOGRAPHY OF POLYNUCLEAR HYDROCARBONS

THE SEPARATION AND ANALYSIS OF POLYNUCLEAR AROMATIC HYDROCARBONS PRESENT IN THE HUMAN ENVIRONMENT

E. Sawicki

The main reason for continuing interest in the analysis for carcinogens in the human environment has been the steadily increasing rate of lung cancer. The group of carcinogens with which all human beings come in contact in varying degrees are the polynuclear hydrocarbons. It is the purpose of this outline to consider the methods and technics known for the separation and analysis of this important group of compounds.

Column Chromatography is usually the first step in the analysis of a solution or an extract of a complex mixture for polycyclic aromatic hydrocarbons. Structural comparison of such compounds has shown that their chromatographic adsorbability on alumina is greater for a) a compound with more rings, e.g., chrysene is adsorbed more strongly than phenanthrene; b) a compound with more double bonds, e.g., benz[a]anthracene is adsorbed more strongly than pyrene; c) an acene (a linearly condensed arene) than for an isomeric phene (an angularly condensed arene), e.g., naphthacene is adsorbed more strongly than benz[a]anthracene; d) fluorenic hydrocarbons than for peri condensed hydrocarbons with the same number of rings and double bonds, e.g., 11H-benzo[b]fluorene is adsorbed more strongly than pyrene; e) the most nearly coplanar compound of a group, e.g., the decreasing order of adsorbability 2-phenylanthracene > 1-phenylanthracene > 9-phenylanthracene follows the increasing angle of twist in these molecules; f) a hydrocarbon substituted with an increasing number of sterically unhindered methyl or alkylene groups than for the hydrocarbon itself, e.g., 9-methylanthracene is adsorbed more strongly than anthracene; and g) the most extensively conjugated isomer, e.g., 1-(2-naphthyl) cyclopentene is adsorbed more strongly than 3-(2-naphthyl) cyclopentene. It is postulated that adsorption on alumina involves π-type complexation where the active sites on the alumina are relatively broad electron-attracting areas to which the electron-donating hydrocarbon substrate is held monomolecularly and preferentially in a planar configuration parallel to the surface if such arrangement is sterically possible.

The behavior of benzo[a]pyrene on various types of partially deactivated silica gel columns with various eluting agents has been studied. Nonpolar developing solvents containing small amounts of acetone or ether were found to give the narrowest elution bands. The separation of petroleum into hydrocarbon groups by means of alumina and silica-gel column chromatography, as well as a similar separation of heavy gas and cycle oils on silica gel, has been described. The behavior of naphthalene and anthracene derivatives on alumina and silica gel has been studied.

The chromatographic properties of some heterocyclic compounds have also been investigated. Polynuclear thiophenes are eluted with hydrocarbons of a similar ring number. Carbazole is eluted directly after coronene. In this case, a ring NH is equivalent to 4 or 5 benzo rings. On the other hand, dibenzofuran and dibenzothiophene are eluted close to fluorene and directly after the bicyclic hydrocarbons.

Details on the standardization of alumina and silica gel for the chromatographic separation of polycyclic aromatic hydrocarbons from airborne particulates are available.

The elutions of compounds of an incinerator effluent sample from an alumina column with pentane-ether mixtures is in the order: sulfur, alkylnaphthalene, phenanthrene and anthracene, pyrene and fluoranthene, 11H-benzo[a]fluorene and 11H-benzo[b]fluorene, triphenylene and dibenzaulenes (?), chrysene and benz[a]anthracene, benzo[ghi]fluoranthene and naphthol [2,1,8,7-klmn]xanthene and benzo[a]naphthacene, benzo[a]pyrene and benzo[e]pyrene and perylene and benzo[k]fluoranthene, benzo[ghi]perylene and anthanthrene, and coronene.

A procedure has been described for the analysis of aromatic hydrocarbons via liquid chromatography of the mixtures under operating conditions analogous to those of gas chromatography. A 23-inch alumina column has been recommended for the separation of polycyclic aromatic hydrocarbons from airborne particulates. The advantages are better separation of the hydrocarbons and inclusion of a few more hydrocarbons in an analysis. The disadvantages are that about 2-3 times as many fractions must be collected and the analysis takes about twice as long.

Polynuclear aromatic hydrocarbons have been separated on alumina with the following solvents: cyclohexane, petroleum ether, petroleum ether with increasing amounts of benzene, petroleum ether with increasing amounts of ether, cyclohexane with increasing amounts of benzene, cyclohexane with increasing amounts of ether, pentane with increasing amounts of ether, cyclohexane with increasing amounts of acetone, petroleum ether with increasing amounts of acetone, and hexane. Benzo[a]pyrene and benzo[ghi]perylene are almost completely separated on the alumina column with pentane-ether mixtures. Usually small amounts (ca. 5%) of the hydrocarbons are found in one intermediate tube.

Pentane has many advantages in column chromatography and subsequent analysis. It is purified easily, evaporates quickly at a low temperature, readily dissolves pentacyclic and smaller aromatic hydrocarbons and many hexacyclic ones, and has a low blank in spectrophotofluorometric analysis. In addition, the absorption spectra obtained can be determined to 220 mμ, and of all known compounds only benzo[a]pyrene has a triplet at 377, 370, and 382 mμ in pentane. The hydrocarbons have more fine structure in their spectra and sharper bands in pentane than can be obtained in cyclohexane or other more polar solvents.

Other useful adsorbents and their eluting solvents are silica gel with cyclohexane followed by carbon tetrachloride or benzene; magnesia-Celite with boiling benzene; silica gel with hexane followed by 4:1 hexane-benzene; and partially acetylated cellulose with 4:17:1 benzene for toluene-ethanol-water. In the separation of polynuclear aromatic hydrocarbons from bio-organic material, a group of chromatographic steps in which calcium hydroxide, Celite, alumina, calcium carbonate, and sucrose are used as adsorbents has been described. The aromatic fraction in petrolatum can be concentrated by passage of 1 kg. in hot isooctane through a silica gel column.

For the analysis of an airborne particulate organic fraction, only a single column separation of 15-150 mg. of material is necessary. The entire operation and ultraviolet-visible spectral analysis requires about 16 man-hours to determine 7-11 polynuclear aromatic hydrocarbons routinely.

Tobacco-smoke tar contains a much smaller concentration of polynuclear aromatic hydrocarbons (ca. 0.2-1 μg. benzo[a]pyrene per g. of tar) than the urban airborne particulate organic fraction (38-3000 μg. benzo[a]pyrene per g. of fraction). For this reason, the analysis of tobacco-smoke tar for polycyclic hydrocarbons is far more difficult. Vacuum distillation, liquid-liquid extraction, and several column and paper chromatographic separations are necessary terminating in ultraviolet-visible spectral analysis. These operations require about 48 man-hours. About 2000-3000 mg. of material is necessary to analyze for three polynuclear aromatic hydrocarbons routinely. An alternative procedure for the analysis of polycyclic hydrocarbons in cigarette-smoke tar consists of five column separations followed by ultraviolet-visible spectral analysis. A detailed account of the analysis of cigarette-tobacco tar has been presented with the identification of 28 compounds via column and paper chromatography and absorption spectra.

The cellulose acetate column is of value in the separation of the larger polycyclic aromatic hydrocarbons. Compounds that are eluted almost simultaneously from an alumina column can be resolved from a mixture. One example is the clear-cut separation of a mixture of benzo[a]pyrene, perylene, benzo[b]fluoranthene, benzo[j]fluoranthene, benzo[k]fluoranthene, and benzo[ghi]perylene. The cellulose acetate column is stated to be most useful following alumina column chromatography.

The following R_f values were obtained for hydrocarbons by elution with 17:4:1 ethanol-toluene-water through a partially acetylated cellulose

column: 9,10-diphenylanthracene, 0.675; acenaphthylene, 0.576; 11H-benzo[b]fluorene, 0.539; 1,1'-dinaphthyl, 0.539; phenanthrene, 0.501; anthracene, 0.494; benzo[c]phenanthrene, 0.483; pyrene, 0.471; triphenylene, 0.446; fluoranthene, 0.441; coronene, 0.410; fluorene, 0.401; benz[a]anthracene, 0.396; 2,2'-dinaphthyl, 0.359; perylene, 0.351; dibenz[a,h]anthracene, 0.246; benzo[b]-fluoranthene, 0.209; benzo[rst]pentaphene, 0.209; chrysene, 0.206; anthanthrene, 0.163; benzo[a]pyrene, 0.093; dibenzo[b,n]perylene, 0.042; and violanthrene, 0.023. This column can resolve compounds having an R_f difference of 0.08 with absolute R_f values > 0.4 and compounds having an R_f difference of 0.05 with R_f values < 0.4. The quality of cellulose acetate can be tested on a column 1 by 12 inches by the elution of 2 mg. of an equimolar mixture of perylene and benzo[a]-pyrene with 17:4:1 ethanol-toluene-water. When the perylene band tail has moved 10 inches, this tail and the benzo[a]pyrene band front should be 6.5-7 inches apart.

In a few instances, aromatic compounds have been concentrated by reaction with a π-acid in solution; the precipitated π-complex is passed through an alumina column to obtain a more highly concentrated fraction of polycyclic aromatic hydrocarbons. 2,4,7-Trinitrofluorenone and picric acid have been used as π-acids in such procedures. Alternatively an organic fraction is passed through a column containing an adsorbent impregnated with a π-acid. Some π-acids that have been used in this fashion are 1,3,5-trinitrobenzene, picric acid, 2,4,5,7-tetranitrofluorenone, and tetrachlorophthalic anhydride. It was observed that with tetranitrofluorenone on an alumina column, the benzpyrene fraction lacks benzo[k]-fluoranthene.

Paper chromatography is an important tool for the separation of polycyclic compounds; reported R_f values, as well as the type of paper and the solvent system, are summarized in Table I. The experimental aspects of ascending paper chromatography have been discussed thoroughly. This technic is usually applied following column chromatography, and yields a purer compound, which can be characterized by ultraviolet-visible absorption spectrophotometry and excitation and emission spectrophotofluorometry. In a final paper-chromatographic step, tracer technics with C^{14}-labeled hydrocarbons have been used to determine benzo[a]pyrene in cigarette-smoke and gasoline-engine exhaust condensates.

The acetylated paper-chromatographic technic has been applied to pyrolysis products, and cigarette-smoke, gasoline-engine-exhaust, and diesel-exhaust condensates. It is claimed that 0.1 μg. of benzo[a]pyrene can be quantitatively separated from 0.5-2.0 g. of diesel-exhaust condensate.

Tarbell's paper-chromatographic procedure has been applied in the analysis of cigarette-smoke tar and the separation of quinones and aromatic hydrocarbons, and the procedure of Bergmann and Gruenwald to the analysis of cigarette-smoke condensate. Many of the systems of separation have been evaluated. The Maly technic has been used to detect and to estimate crudely a few atmospheric polycyclic aromatic hydrocarbons by measurement of the horizontal spot diameter.

The aqueous acetic acid technic has been applied in studies of polycyclic hydrocarbon synthesis during incomplete combustion. An 83% aqueous formic acid solution has been claimed to be one of the best developing solvents on S and S 2043b paper for the separation of polynuclear aromatic hydrocarbons. Benzo[a]pyrene and benzo[ghi]perylene are easily separated.

Because methylation of many polycyclic hydrocarbons can lead to increased carcinogenic activity, the separation, characterization, and determination of alkyl derivatives of the polycyclic hydrocarbons is important. The final tool in the separation has been paper chromatography. The alkyl derivatives are characterized mainly by R_f value and ultraviolet-visible absorption spectra. Obviously, the identification of the alkyl group and, sometimes, its position is uncertain.

Anthracene, 1-methylanthracene, 2-methylanthracene, 2,7-dimethylanthracene, and 1,4-dimethylanthracene have been separated on propylene glycol-impregnated paper with toluene-methanol as the developer, and on acetylated paper with 2:10:2 petroleum ether-methanol-water. Fluorene has been readily separated from its 1-, 2-, and 3-methyl derivatives on propylene glycol-impregnated paper with 85:10:5 methanol-water-acetic acid. A study of the separation of benzo[c]phenanthrene, benz-[a]anthracene, and eleven of their monomethyl derivatives has disclosed that in most cases the position of substitution can be assigned unequivocally. Benzo[a]-pyrene and its 6-ethyl, 6-acetyl, and 6-(a-hydroxyethyl) derivatives are easily separated. Benzo[a]pyrene and 5-methyl-benzo-[a]pyrene have been separated, as have benzo[a]pyrene and its 2- and 6-methyl homologs. Pyrene and its methyl derivatives can be separated on Whatman No. 1 paper oracetylated paper.

The developed spots or bands are cut out and extracted in a micro Soxhlet extractor, or other simple micro extraction apparatus with solvents such as 4:1 benzene-alcohol under nitrogen. hexane-methanol and ether.

Losses during chromatography can be expected. In the chromatography of cigarette-smoke condensate, twice on a silica gel column and four times on paper, 35-40% of the benzo[a]pyrene was lost. It has been reported that up to 20 mg. of material can be fractionated on a 28 by 50 cm. sheet of paper.

To prevent photodegradation of material on the paper, the room light sources are covered with yellow butyrate plastic (Tenite), which absorbs all radiation below 470 mμ. Another convenience is the automatic addition funnel described by Van Duuren.

Other separations that have been studied are fluoranthene and its methyl derivatives, chrysene and 6-methylchrysene, and perylene and 3-methylperylene. Via column and paper chromatography, followed by absorption and fluorescence spectrophotometry, 4-methylpyrene and 8-methylfluoranthene, and alkylated chrysene, fluoranthene and pyrene have been found in cigarette-smoke condensate. Alkylated derivatives of chrysene, pyrene, fluoranthene. Benz[a]anthracene, and benzo[a]pyrene have been found in gasoline-engine-exhaust condensate. 9-Methyl-anthracene is readily separated from anthracene by paper chromatography and thereby has been found in polluted air.

Since so many polycyclic aromatic hydrocarbons have been found in combustion products, polluted air, etc., the presence of fluorenonic, benzanthronic, and quinonic compounds would be expected; anthanthrone and a benzo[a]-pyrene quinone have been reported in polluted air. Paper chromatography of fluorenone with 40% aqueous acetic acid gave an R_f value of 0.91, identical to that of anthracene. As shown in Table I, many quinones can be readily separated from each other or from their parent hydrocarbons by either of two developing systems.

Detection of Spots in Paper Chromatography. Fluorescence colors on paper have been reported for many polynuclear aromatic hydrocarbons and some derivatives.

Some of the smaller of these hydrocarbons fluoresce on paper when excited by short-wavelength ultraviolet illumination. The sensitivity of the fluorescent test is within the range 0.1 to 0.001 μg.

Color tests on paper for various hydrocarbons have been reported with tetrachlorophthalic nahydride, picric acid, 2, 4, 7-trinitrofluorenone, and tetracyanoethylene. The last reagent give a wider range of colors than do the others and also yields brilliant colors with heterocyclic compounds. 2, 4, 5, 7-Tetranitrofluorenone could also be tried in the characterization of the spots. The piperonal chloride test has been applied on paper and gives a wide range of colors with aromatic compounds. For most polynuclear hydrocarbons, these color reactions are much less sensitive than the fluorescence colors on paper. Many of the colors fade. In the tetracyanoethylene test, the colors fade in 3-60 min. In the piperonal test, the colors fade rapidly, but can be revived by the addition of trifluoroacetic acid.

Fluorescence spectra can be obtained directly from paper; explicit directions have been given to obtain qualitative and quantitative date. With increasing concentration, some of the spectra change drastically, and in some cases a red spectral shift results. This phenomenon may be related to the fluorescent changes of dilute solutions of some hydrocarbons as compared to their microcrystalline state, and should be studied further for possible analytical application.

Thin-layer chromatography has been applied to only a small extent for the separation of polynuclear aromatic hydrocarbons from complex mixtures, but will find increased use. The advantages of this technic are that it is simple, rapid, inexpensive, versatile, and sensitive, requires little working material, and can resolve complex mixtures. It is particularly suitable for studying air-borne particulates. The results obtained with paper and column chromatography can be applied to thin-layer chromatography. A novice can secure good separations the first day.

Azulene has been separated on thin-layered silica gel with hexane-ethyl acetate as the developing solvent. On an alumina-coated plate, cyclohexane separates polynuclear aromatic hydrocarbons in the ascending order: coronene, benzo[ghi] perylene, benzo[a]-pyrene, benzo[e] pyrene, fluoranthene, and pyrene. Three hours each were required for equilibration and for a run. Fourteen distinct fluorescent zones were obtained in 24 hours through thin-layer chromatography of an acetone extract of atmospheric dust collected on the filter of an air conditioner.

Thin-layer chromatography has been shown to work well for the separation of fluorescent polynuclear compounds. Although alumina with 19:1 pentane-ether was found to give poor separation of individual polycyclic hydrocarbons, it gave the best results with complex high-boiling mixtures. Over 24 fluorescent spots could be obtained with the benzene extract of air-borne particulates. This adsorbent was found to work best for the separation of classes of aromatic compounds. Cellulose with aqueous organic solvents gave the best separations of individual polynuclear hydrocarbons, while cellulose acetate with ethanol-toluene-water gave the best separation of benzo[a] pyrene from other hydrocarbons. This last adsorbent has been applied to the separation of benzo[a] pyrene and benzo[k]-fluoranthene from each other and from the remainder of the polycyclic hydrocarbons in air-borne particulates. A large number of systems have been devised for the separation of aza hydrocarbons from each other or from other types of polynuclear compounds. Cellulose acetate plate have been used to separate polycyclic hydrocarbons, and silica gel with 1:1 hexane-benzene for the separation of simpler polynuclear compounds according to class. High boiling compounds not completely resolved by gas chromatography can sometimes be elucidated by thin-layer chromatography of the trapped fractions.

Thin-layer chromatographic separation of classes of compounds or of individual compounds should prove of great value in trace analysis. This technic in conjunction with spectrophotofluorometry, spectrophosphorimetry, and high-temperature gas chromatography should allow analysis at the nanogram and picogram level. Some of the thin-layer systems that have been used are summarized in Table II.

Table II

THIN-LAYER CHROMATOGRAPHY OF POLYCYCLIC HYDROCARBONS

Adsorbent; solvent system (reference)[a]	R_f values[b]
cellulose, 50% aq. DMF	Hydrocarbons ranging from Phl, 0.66 to DBb, rstPP, 0.046.
cellulose, acetylated; 4:4:1 MeOH-Et_2O-H_2O	DBdef, pC, 0.56; N1, 2, 3, 4,-defC, 0.50; PER, 0.34; BkFT, 0.23; BrstPP, 0.22; BbFT, 0.15; BaP, 0.11.
same; 17:4:4 EtOH-T-H_2O	Hydrocarbons ranging from Phl, 0.53 to BaP, 0.14.
alumina; 19:1 Pen-Et_2O	Hydrocarbons ranging from Phl, 0.96 to BaCOR, 0.085.
same; Hex	IE, 0.67; N, 0.63; DPL, 0.58; 1-Me'N, 0.53; 2-Me'N, 0.52; 2, 6-DMe'N, 0.52; BbTH, 0.50; 1, 5-DMe'N, 0.48; 1, 6-DMe'N, 0.48; AC, 0.44; 2, 7-DMe'N, 0.42; 2, 3-DMe'N, 0.39; F, 0.35; A, 0.35; Phl, 0.20; DT, 0.18; DF, 0.11; FT, 0.10; P, 0.10; C, 0.00; BaP, 0.00; 9, 9'-D'A, 0.00; I, 0.00; CAR, 0.00; Q, 0.00.
same; CCl_4	N, 0.85; 2-Me'N, 0.85; 1, 5-DMe'N, 0.83; 2, 6-DMe'N, 0.85; 1, 6-DMe'N, 0.83; BbTH, 0.83; 2, 3-DMe'N, 0.81; IE, 0.81; 2, 7-DMe'N, 0.80; DPL, 0.79; 1-Me'N, 0.78; F, 0.75; DF, 0.73; AC, 0.70; DT, 0.67; Phl, 0.66; P, 0.65; A, 0.59; FT, 0.55; BaP, 0.10; 9, 9'-D'A, 0.06; I, 0.05; CAR, 0.05; C, 0.00; Q, 0.00.
silica gel; 1:1 Hex-B	F, 0.33; 1-Me'A, 0.49; A, 0.48; BCAR?, 0.48; CAR, 0.55; 5-Me'I, 0.52; 6-Me'Q, 0.07; ACR, 0.06; 1-Me'ACR, 0.05.
same; Hex	N, 0.59; IE, 0.55; 1-Me'N, 0.54; 1, 6-DMe'N, 0.54; 2, 7-DMe'N, 0.53; BbTH, 0.53; 2-Me'N, 0.50; 1, 5-DMe'N, 0.50; 2, 6-DMe'N, 0.50; 2, 3-DMe'N, 0.48; DPL, 0.45; AC, 0.44; DBTH, 0.42; DF, 0.39; A, 0.39; Phl, 0.33; F, 0.32; P, 0.32; BaP, 0.17; 9, 9'-D'A, 0.10; I, 0.06; C, 0.00; CAR, 0.00; Q, 0.00.
same; CCl_4	2-Me'N, 0.87; IE, 0.84; AC, 0.83; N, 0.77; 1, 6-DMe'N, 0.75; 2, 3-DMe'N, 0.75; 1-Me'N, 0.74; DPL, 0.74; BbTH, 0.73; DF, 0.73; 1, 5-DMe'N, 0.72; 2, 6-DMe'N, 0.72; DT, 0.70; F, 0.66; FT, 0.66; A, 0.65; Phl, 0.65; BaP, 0.65; P, 0.64; 9, 9'-D'A, 0.58; CAR, 0.15; I, 0.12; C, 0.11; Q, 0.00.

a—— Same abbreviations as footnote a, Table I.
b—— Same abbreviations as footnote b, Table I.

Detection and Characterization of Spots in Thin-Layer Chromatography by extremely sensitive color, fluorescent, cold-fluorescent, and phosphorescent tests has been reported and applied to the analysis of air pollution source and airborne particulates. Some of the reagents used successfully for characterization include piperonal, trifluoroacetic acid, sulfuric acid, 2, 4, 5, 7-tetranitrofluorenone, tetracyanoethylene, and tetraethylammonium hydroxide. The use of tetracyanoethylene and formaldehyde-sulfuric acid has been described in the characterization of 30 polynuclear aromatic and heterocyclic hydrocarbons on thin-layer plates. Many non-fluorescent compounds can be made to fluoresce or phosphoresce strongly at low temperatures; this phenomenon allows development of extremely sensitive procedures for characterization or determination, which should be of value for polynuclear aromatic hydrocarbons.

I ANALYSIS

For the identification and determination of polynuclear hydrocarbons, methods based on ultraviolet-visible absorption spectra, fluorescence spectra, and phosphorescence spectra are prominent. Electron-spin resonance has promise with less than 10^{-10} mole of the hydrocarbons being detectable. Spot tests in paper and thin-layer chromatography were treated earlier in this review.

A Absorption Spectra of chromatographically-separated unknown compounds are invaluable for their characterization. The advantages of absorption spectra (and the disadvantages of fluorescence spectra) lie in that the wavelength maximum of a compound is unaffected by the presence of other compounds, quenching effects are absent, and absorption curves of many polynuclear aromatic hydrocarbons are available in the literature. The main disadvantage, compared to spectrophotofluorometry, is the lower sensitivity. The last step in the determination of hydrocarbons in some natural mixtures is usually absorption spectrophotometry.
Most of the papers referred to in this section make use of a baseline method of measurement, as described by Cooper and Commins. References have been compiled on the analysis of hydrocarbons in polluted air and tobacco smoke as performed by researchers at Sir John Cass College.

Absorption bands are useful in the characterization of polycyclic hydrocarbons found in combustion products. Some of the long wavelength bands are useful in the determination of these hydrocarbons.

Collections of absorption spectra and spectral data are available: references to spectra of 370 hydrocarbons, continuing collection of spectral data, collection of 370 curves, largely polynuclear compounds, longest wavelength band of 408 polycyclic aromatic hydrocarbons with 143 and of 204 polynuclear heterocyclic compounds with 70, data for 42 polynuclear aromatic hydrocarbons in pentane, data for methylantracenes, methylphenanthrenes, and methylbenzo[c]phenanthrenes in heptane at room temperature and pentane at —100°, data for benz[a]anthracene and its methyl derivatives in heptane at room temperature and pentane at —100° and in alcohol, and basicity constants and data for these compounds in anhydrous hydrogen fluoride, curves and basicity constants for 36 polycyclic hydrocarbons and quinones in sulfuric acid, curves for about 80 polynuclear aromatic hydrocarbons, and curves for anthracene derivatives. Absorption spectra and empirical correlations for many hydrocarbons have been reported by Clar over the past 35 years.

Photometric methods of analysis for polynuclear hydrocarbons have been reviewed. The advantages and disadvantages of the piperonal method for the determination of benzo[a]pyrene in air have been enumerated with a statistical study of 200 airborne particulate samples.

The 3-methyl-2-benzothiazolinone photometric methods for azulenes and imino heterocyclic derivatives are sensitive, and the latter have been applied to the determination of carbazole in samples of air polluted with coal-tar pitch. Various methods for the determination of azulene have been evaluated. Bromination has been applied to the analysis of pyrene and iodination of benzo[a]pyrene. By the absorption and fluorescent spectra in neutral and acidic solution, some basic and acidic polycyclic compounds can be differentiated and characterized.

B Fluorescence Spectra are important aids in the characterization and determination of polynuclear compounds, for each of which excitation and emission spectra can be obtained. The excitation spectrum of a fluorescent hydrocarbon resembles its absorption spectrum. Consequently, an excitation spectrum can serve for the identification of a compound, even with nanogram samples, if the absorption spectra were previously established. In addition, it is possible to determine quickly whether a chromatographic spot is a mixture of fluorescent compounds. If different emission spectra are obtained at different excitation wavelengths, then the spot is a mixture.

For many fluorescent compounds the excitation and emission spectra are 10 to 10,000 times more sensitive than its absorption spectrum. For example, the identification limit for benzo[a]pyrene in pentane is 2500 nanograms (λ = 0.1) in a 3-ml. Cary absorption spectrophotometric cuvet and 0.25 nanogram in a 0.1-ml. Aminco-Bowman spectrophotofluorometric cell. Consequently, an extract of a spot containing 5 nanograms of benzo[a]pyrene will give substantial fluorescence spectra, but no absorption spectrum. The disadvantages of spectrophotofluorometry as compared to absorption spectrophotometry are the poorer reproducibility, the fewer available quantitative spectra, and quenching, which can drastically affect the sensitivity and shape of the spectral curve.

Occasionally fluorescence analysis has been used as the first step in the determination of polycyclic hydrocarbons in mixtures after column or paper chromatography, especially in the pioneering studies, but also in more recent, refined ones. Much of this work was conducted before it was known that benzo[k]fluoranthene had a fluorescence spectrum similar to that of benzo[a]pyrene. With the further perfection of spectrophotofluorometers, the future should see the development of reliable, ultrasensitive methods for the determination of the polynuclear hydrocarbons. Benzo[a]pyrene has been characterized and determined from its fluorescence line spectrum at —196° in octane. The method is sensitive to 1 nanogram per ml. The use of non-polar solvents at very low temperatures should confer greater selectivity on the method. For example, benzo-

[ghi] perylene, which exhibits 4 bands in its fluorescence emission spectrum in pentane at room temperature, has 28 bands in hexane at —180° and 74 maxima when the spectrum is recorded photographically. At lower temperatures, perylene is determined more readily in crude oil.

Benzo[a] pyrene exhibits selective and sensitive excitation and emission spectra in sulfuric acid. This property has been made use of to determine minute amounts of benzo[a] pyrene in polluted air samples following thin-layer chromatography on alumina. Sample weights of 0.2 mg. could be analyzed. Benzo[k] fluoranthene, benzo[ghi] perylene, perylene, benzo[e]-pyrene, and anthanthrene do not interfere.

Fluorescence excitation and emission spectra have been found to be invaluable in the characterization of polycyclic hydrocarbons separated by chromatography.

Ozone in the fluorometric determination of benzo[a] pyrene and laboratory illumination on polycyclic hydrocarbons have been shown to lead to decomposition of the hydrocarbons.

As the fluorescence emission spectra of benzo[a] pyrene and benzo[k] fluoranthene are similar in neutral solvents at room temperature, methods of chemical differentiation or separation are used to help characterize or determine either of the two compounds. As bromine quenches the fluorescence of benzo[a] pyrene without affecting that of benzo[k] fluoranthene, either compound can be determined fluorometrically. The shift in spectrum of compounds in microcrystalline suspension as compared to their solution has been used. In aqueous acetic acid, the fluorescence of benzo[a] pyrene disappears completely from the 400 to 500 mμ region, whereas that of benzo[k] fluoranthene remains virtually unchanged.

Fluorescence excitation and emission spectra have been recorded for many polynuclear aromatic hydrocarbons: emission spectra of 36 hydrocarbons, excitation and emission spectra of 41 hydrocarbons in pentane and 17 in concentrated sulfuric acid, emission spectra on paper of 18 hydrocarbons, emission spectral curves and wavelength maxima of 45 hydrocarbons and their methyl derivatives in isooctane, emission spectra of about 70 polycyclic aromatic and aza heterocyclic compounds in light petroleum ether, emission spectra of many compounds in thin solid films, emission spectra in hexane of 6 polycyclic hydrocarbons, and emission spectra of 44 hydrocarbons and 10 imino and aza heterocyclic compounds in cyclohexane.

It is sometimes possible to obtain the pure fluorescence emission (or excitation) spectrum of an aromatic hydrocarbon by determining the spectrum of the mixture at an appropriate excitation (or emission) wavelength maximum of that hydrocarbon. Factors that alter the fluorescence of polynuclear hydrocarbons have been investigated. A review with 187 references covers the effect of solvent and concentration changes, complexing, and low temperatures on the fluorescence of aromatic compounds in solution.

C Phosphorescent Spectra have as yet seen little use in trace analysis, but should be found to be invaluable for the following reasons. Many compounds that are non-fluorescent or weakly so are intensely phosphorescent, for example, triphenylene, chrysene, naphthalene, fluorene, dibenzothiophene, carbazole, picene, coronene, benzo[e] pyrene, phenanthrene, biphenyl, 4H-cyclopenta[def] phenanthrene, many aryl ketones, quinones, nitroarenes, and aryl sulfones. Ultraviolet-visible absorption and fluorescence spectral technics work best for compounds absorbing at the longer wavelengths as compared to other compounds in the mixture. The short-wavelength bands are rarely used in characterization or determination of a compound in a mixture. However, many compounds that are not studied because they absorb at short wavelengths, where a large amount of interference is present, can be analyzed through their phosphorescence spectra. Thus, for polycyclic hydrocarbons having para-bands below 370 mμ, phosphorescence spectral technics should prove to be of great value. The quantum yield of phosphorescence has been reported to decrease with increasing wavelength of the para band of the hydrocarbon. Mixtures containing more than two phosphorescent substances can be analyzed in some instances by the proper choice of excitation frequency, resolution time, and emission frequency.

Phosphorimetric analysis is performed in rigid media. Organic solvents that freeze to an unbroken glass (e.g., under liquid nitrogen) usually serve as the test solvent, and have been listed. Propylene glycol-water has been recommended for water-soluble compounds. These same solvents and freezing liquids can be used also in low-temperature fluorescence analysis.

Lewis and co-workers investigated the phosphorescent state in several classical studies that are proving to be of value to the analyst. Spectrophosphorimetric analysis should become an important tool in the field of trace analysis of mixtures following chromatography.

The phosphorescence spectra of benzo[a]pyrene, biphenyl, phenanthrene, fluorene, dibenzofuran, and phenanthro[3,4-c]phenanthrene have been reported. The phosphorescence spectra of many polynuclear aromatic hydrocarbons are available: emission spectra of 32 hydrocarbons correlated with their chemical reactivity and para-absorption bands, emission spectra of benz[a]-anthracene, benzo[c]phenanthrene and their methyl derivatives and the corresponding spectra of their 1,3,5-trinitrobenzene complexes, low-temperature fluorescence and phosphorescence emission spectra of benz[a]anthracene, benzo[c]-phenanthrene and their methyl derivatives emission spectra of 10 hydrocarbons and reference to 35 others, the low-temperature fluorescence and phosphorescence emission spectra of coronene, benzo-[ghi]perylene, perylene, triphenylene, 9,10-dichloroanthracene in crystalline paraffins at —180°, and a review of the excitation and emission spectral data of about 240 aromatic compounds.

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